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A STUDY OF THE REINNERVATION OF STRETCH RECEPTORS

IN CAT MUSCLE FOLLOWING NERVE INJURY

A thesis presented in candidature for the degree

Doctor of Philosophy

by

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Durham, April 1983.

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28. NOV 1983

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Abstract

An histological investigation into the reinnervation of stretch receptors necessarily requires a reliable staining technique. To achieve this, silver staining has been investigated and standardised. The use of a diffusion-limiting barrier during the impregnation stage has greatly improved the quality and extent of staining.

The process of reinnervation has been examined in the peroneal muscles of the cat following different types of nerve injury, including crush, section and freeze.

After nerve crush, most motor and sensory axons form endings of a recognisable form in the usual positions on muscle spindles and tendon organs.

The late arrival of some sensory axons, and aberrant motor formations on the poles of spindles, are attributed to the fact that large axons are damaged more than small axons during the nerve crush. It is suggested that some of these motor axons may have previously supplied only extrafusal muscle fibres.

Although abnormalities in the restoration of the primary ending are common, none is sufficiently consistent to explain the abnormalities in the responses of reinnervated spindles. This implies that these may be caused by a maturing transduction mechanism.

The fact that after nerve section the restoration of spindle innervation is poor, whereas after nerve freeze (during which damage to supporting tissue is minimised), it is close to normal, indicates that physical guidance plays an important role in the reinnervation process.

It is argued that, after short periods of denervation, muscle spindles show a marked " site-type " specificity of sensory reinnervation. This is seen to diminish after longer periods of denervation, possibly being influenced by the fusimotor innervation.

CONTENTS

Acknowledgements	i
Abstract	ii
Contents	iii

CHAPTER ONE: INTRODUCTION

SECTION ONE: THE STRUCTURE AND PHYSIOLOGY OF THE MUSCLE SPINDLE

INTRAFUSAL MUSCLE FIBRES	1
1) Chain fibres	2
2) The bag ₁ fibre	4
3) The bag ₂ fibre	5
THE SENSORY TERMINALS	6
1) The primary ending	6
2) The secondary ending	7
THE AFFERENT INNERVATION	8
THE AFFERENT RESPONSE	9
THE MOTOR TERMINALS	11
THE EFFERENT INNERVATION	12
1) The fusimotor (gamma) innervation	12
2) The skeletofusimotor (beta) innervation	14

SECTION TWO: MUSCLE SPINDLE REINNERVATION

INTRODUCTION	17
EFFECT OF DENERVATION AND REINNERVATION ON THE SPINDLE	17
RESTORATION OF TERMINALS	22
PHYSIOLOGICAL RECOVERY AFTER NERVE LESIONS	23
FUNCTIONAL RECOVERY OF MUSCLE SPINDLES AFTER NERVE LESIONS	23
1) Afferent response	24
a) Primary responses	24
b) Secondary responses	24

FUSIMOTOR REINNERVATION	26
NATURE OF LESION AND DIFFERENTIAL RATES OF RETURN	27
THE PRESENCE OF ABERRANT MOTOR AXONS	29
SPECIFICITY OF REINNERVATION	30
CONCLUDING REMARKS	31

CHAPTER TWO: STAINING TECHNIQUE

STAINING TECHNIQUE	32
1) Theoretical basis of silver staining	34
a) Silver neurohistological methods	34
i) "Reducible" silver	38
b) Development	40
c) Summary	42
2) Standardisation procedures	45
a) Dissection procedure	45
b) Position of muscle within solution	46
c) Ratio of volume of muscles to volume of solutions	46
d) Glassware	46
3) Investigations	48
CONCLUDING REMARKS	55

CHAPTER THREE: THE CRUSH STUDY

EXPERIMENTAL DESIGN	56
PRELIMINARY EXPERIMENTS	57
ANALYSIS	60
1) Identification of intrafusal fibre type	60
2) Identification of nerve terminals	61
a) Identification of sensory terminals	61
i) Primary endings	61

ii) Secondary endings	61
b) Identification of motor terminals	62
The gamma innervation	62
i) Trail innervation	62
ii) The p_2 plate innervation	62
c) The skeletofusimotor (beta)	
innervation	63
i) The p_1 plate innervation	63
NERVE DIAMETER MEASUREMENTS	64
HYPERINNERVATION	64
1) Motor terminals	64
2) Sensory terminals	64
OBSERVATIONS AND RESULTS	65
SENSORY REINNERVATION	65
1) Restoration of primary endings	65
a) Overview of restoration	65
b) The rate of return	67
c) The diameter of Ia axons	67
d) Hyperinnervation	67
e) Distribution of reinnervated primary	
endings to intrafusal muscle fibres	68
f) Abnormalities of reinnervation	68
g) Position and extent of the primary	
ending	69
h) Other axons in the equatorial	
region	69
2) Restoration of secondary endings	71

a) Rate of return	71
b) Diameter of II axons	71
c) Hyperinnervation	71
d) The form of the ending	71
e) Position and extent of endings	72
f) Interactions with other axons	72
MOTOR REINNervation	73
RESTORATION OF MOTOR ENDINGS	73
1) Restoration of extrafusal endplates	73
2) Restoration of p_1 plates	74
a) Overview of restoration	74
b) Diameter of beta axons	74
c) The usual pattern of restoration of p_1 endplates	74
d) Hyperinnervation	75
3) Restoration of p_2 plates	76
a) Overview of restoration	76
b) The diameter of gamma axons supplying p_2 plates	76
c) The length of p_2 plates	76
d) Hyperinnervation	76
e) Other abnormalities	77
4) Trail terminals	77
5) Visiting axons	78
RESTORATION OF GOLGI TENDON ORGANS	79
PACINIFORM CORPUSCLES	79
SUMMARY AND COMMENT	80

CHAPTER FOUR: SUPPLEMENTARY STUDIES

SECTION ONE: THE REINNERVATION OF MUSCLE SPINDLES AFTER NERVE SECTION

INTRODUCTION	84
METHODS	84
OBSERVATIONS AND RESULTS	86
SENSORY RESTORATION	86
MOTOR RESTORATION	90
1) Overview of restoration	90
2) Restoration of extrafusal endplates and p_1 plates	90
3) Restoration of p_2 plates and trail endings	91
4) Invading axons	91
5) Restoration of Golgi tendon organs	92
SUMMARY AND COMMENT	93

SECTION TWO: THE REINNERVATION OF MUSCLE SPINDLES BY SENSORY AXONS IN THE

ABSENCE OF MOTOR INNERVATION

INTRODUCTION	94
METHODS	94
OBSERVATIONS AND RESULTS	94
SUMMARY AND COMMENT	95

SECTION THREE: THE REINNERVATION OF MUSCLE SPINDLES IN THE ABSENCE OF

SENSORY INNERVATION

INTRODUCTION	97
EFFICACY OF OPERATIONS	98
OBSERVATIONS AND RESULTS	98
SUMMARY AND COMMENT	99

SECTION FOUR: THE REINNERVATION OF MUSCLE SPINDLES AFTER NERVE FREEZE

INTRODUCTION	100
METHODS	101
PRELIMINARY PROCEDURES	101
THE CALCULATION OF REINNERVATION TIME FOR THE FREEZE LESION	101
ANALYSIS	101
OBSERVATIONS AND RESULTS	102
THE SENSORY INNERVATION	102
1) Restoration of primary endings	102
a) Rate of return	102
b) Diameter of Ia axons	102
c) Hyperinnervation	102
d) Distribution of endings to intrafusal fibres	102
e) Form of the ending	102
f) Position and extent	103
2) Restoration of secondary endings	104
a) Rate of return	104
b) Diameter of group II axons	104
c) Hyperinnervation	104
d) The form of the ending	104
e) Distribution of the endings	104
f) Position and extent	104
g) Interaction with other axons	105
THE MOTOR INNERVATION	106
1) Restoration of extrafusal motor endplates and p_1 plates	106
a) Diameter of Ia axons	106
b) Hyperinnervation	106
2) Restoration of p_2 plates	107
a) Rate of return	107
b) Diameter of axons supplying p_2 plates	107
c) Distribution of p_2 plates	107

d) Abnormalities in p_2 plates	107
3) Restoration of trail ramifications	108
4) Visiting axons	108
GOLGI TENDON ORGANS	110
1) Rate of return	110
2) Nature of the endings	110
3) Diameter of the axons	110
4) Abnormalities	110
 <u>EIGHT WEEK POST-FREEZE MATERIAL</u>	
SENSORY INNERVATION	111
1) Restoration of primary endings	111
2) Restoration of secondary endings	112
MOTOR INNERVATION	112
1) Restoration of extrafusal endplates and p_1 plates	112
2) Restoration of p_2 plates	112
3) Restoration of trail endings	113
4) Invading axons	113
RESTORATION OF GOLGI TENDON ORGANS	113
SUMMARY AND COMMENT	113

SECTION FIVE: THE REINNERVATION OF MUSCLE SPINDLES AND GOLGI TENDON ORGANS

AFTER A MINIMUM PERIOD OF DENERVATION

INTRODUCTION	115
METHOD	115
OBSERVATIONS AND RESULTS	116
SENSORY RESTORATION	116
1) Primary restoration	116
a) Form of the ending	116
2) Secondary restoration	116
MOTOR RESTORATION	116
1) Extrafusal endplates and p_1 plates	116

2) Restoration of p ₂ plates	117
3) Restoration of trail endings	117
4) Invading axons	117
GOLGI TENDON ORGANS	117
SUMMARY AND COMMENT	117

CHAPTER FIVE: DISCUSSION

THE IDENTIFICATION OF REINNERVATING NEURONES	119
SPECIFICITY OF SENSORY REINNERVATION	122
SENSORY NERVE SPROUTING	129
SPECIFICITY OF MOTOR REINNERVATION	130
MOTOR NERVE SPROUTING	132
MECHANISMS OF SPECIFICITY	135
INTRAFUSAL FIBRE SPLITTING	137
GROWTH RATES OF AXONS	139
REINNERVATING AXONS	142
REINNERVATED SPINDLE MORPHOLOGY	143
COMPARISON WITH PREVIOUS REINNERVATION STUDIES	144
SUMMARY	149

APPENDIX

TECHNIQUES

a) Holmes Silver	151
b) Freeze equipment	152
c) Anaesthetic box	153
d) Plastic embedding	155
e) Electron microscopy	157
f) Photography	157
g) Tracing	157

h) Silver staining	158
i) Teasing	160
j) Operating procedure	160

BIBLIOGRAPHY	161
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CHAPTER ONE

CHAPTER ONE

INTRODUCTION

THE STRUCTURE AND PHYSIOLOGY OF THE MUSCLE SPINDLE

The mammalian muscle spindle consists of a bundle of intrafusal muscle fibres, the central part of which is enclosed within a fluid-filled capsule. It receives both sensory and motor innervation and is distributed throughout the majority of somatic muscles.

The morphology and physiology of the spindle have been the subject of several recent reviews: Barker (1974); Hunt (1974, 1978); Kennedy, Poppele & Quick (1980); Laporte (1978) and Matthews (1972, 1980, 1981).

The work presented here is a histological study of spindle reinnervation and therefore the following review will deal mainly with spindle morphology.

Intrafusal muscle fibres

The muscle spindle has been recognised as having two types of intrafusal muscle fibre for some time; Sherrington (1894) recognised two populations of intrafusal fibres, distinguishing between the two mainly on the basis of diameter. Cooper & Daniel (1956) also divided intrafusal muscle fibres into two types, basing this division on difference in fibre length, fibre diameter and in the equatorial nucleation. The two types of fibre have been termed nuclear bag (Barker, 1948) and nuclear chain (Boyd, 1960 , Cooper & Daniel, 1956), because of the disposition of their equatorial nucleation.

That there are more than two types of intrafusal fibre had also been recognised histologically (Barker & Gidumal, 1961), and histochemically (Ogata & Mori, 1962), but there was some confusion as to the exact histochemical and ultrastructural profile, nomenclature and



species distribution of the three kinds of intrafusal fibre. Yellin (1969) reported three types of fibre in the rat, distinguished histochemically, which are equivalent to the glycolytic (type A), oxidative (type B) and oxidative-glycolytic (type C) of extra-fusal muscle fibres.

James (1971), for the rat, and Banks (1971), for the rabbit, termed the three types 1, 2 and 3 using histological criteria, whereas Barker & Stacey (1970), and subsequently Barker, Harker, Stacey & Smith (1972) described three types of intrafusal fibre in the rabbit, based on histochemical, morphological and E.M. studies, naming them typical bag, intermediate bag and chain fibres.

Milburn (1973) described the typical bag, intermediate bag and chain fibres in the rat, using histochemical and morphological techniques, and Ovalle & Smith (1972) distinguished between bag₁, bag₂ and chain fibres in the cat on differences in the myofibrillar ATPase profile. The situation was clarified by Banks, Barker, Harker & Stacey (1975) and Banks, Harker & Stacey (1977), who applied ultrastructural and histochemical techniques to the same spindle. The nomenclature adopted was that proposed by Ovalle & Smith (1972), namely, bag₁, bag₂ and chain fibres.

1) Chain fibres

It was shown that chain fibres have the smallest diameter of the three fibre types in the cat and the rat, and that they are also the shortest fibres, although a " long chain " fibre is present in some cat spindles (Barker, Banks, Harker, Milburn & Stacey, 1976). (The subdivision of chain fibres will be discussed later.) They also established that chain fibres are the most homogeneous of the intrafusal fibres, having an M-line throughout

their length and a high alkaline ATPase profile. However Saito, Tomanaga, Hirayana & Narabayashi (1977) report the occasional presence of a chain fibre with a low alkaline ATPase level, though these were few in number, and were observed only in man.

Chain fibres stain fairly consistently for glycogen (P.A.S.) and phosphorylase (Barker et al., 1976a), whereas there is a light stain with acid ATPase (Kucera, Dorovini-Zis & Engel, 1978).

There are usually several chain fibres present in the cat muscle spindle; the exact number varies with the muscle. Peroneus digiti quinti has on average six (Harker, Jami, Laporte & Petit, 1977), and tenuissimus has on average four (Barker, Emonet-Dénand, Harker, Jami & Laporte, 1976).

Developmentally, they are formed last of the three types of fibre (Milburn, 1973).

It has been recognised for some time that there is more than one type of chain fibre, and that a " long chain " intrafusal fibre can be identified on the criteria of size, motor nerve supply and histochemical profile (Barker et al., 1976a, Harker et al., 1977 , Laporte, 1978 , Jami et al., 1978, 1979 , Kucera, 1980). More recently Kucera (1980) has distinguished between long chain, intermediate chain and typical chain fibres.

"Typical" chain fibres are the most common in cat spindles. They only extend a short distance, if at all, past the capsule limits, and their NADH-TR staining is intense (Kucera, 1980). " Long chain " fibres; those that extend for 1000 μ m or more beyond the end of the spindle capsule, have a less intense NADH-TR staining, at least in the extracapsular region.

" Intermediate " chain fibres resemble long chains in their NADH-TR staining intensity, but they terminate at less than 1000 μ m

past the capsule boundary.

It has been shown (Kucera, 1982a) that long chain fibres are frequently positioned within the layer of chain fibres furthest away from the bag₂ fibre, and that they are usually associated with the bag₁ fibre. Intermediate chain fibres have been shown to adopt similar positions to the long chains. Kucera (1982a) interprets the relative positioning of the fibres as reflecting the developmental sequence, with the long chain being the first of the chain fibres to be formed, following the formation of the bag₁ fibre with which it remains associated.

2) The bag₁ fibre

As the distinction between the two types of bag fibre relies in part on different histochemical and ultrastructural profiles, Barker et al. (1976a) found it convenient to distinguish three regions between the equator and the origin of a spindle pole. These are region A, that part of the equatorial region lying between the equator and the equatorial end of the periaxial space; region B, that part of the pole extending from the equatorial end of the periaxial space to the end of the capsule; and region C, the extracapsular part of the pole.

Usually there is only one bag₁ fibre per spindle, but more have been reported (Saito et al., 1977 , Barker et al., 1976a, Banks, Barker & Stacey, 1979 & 1982), and spindles have been found which do not possess a bag₁ fibre (Banks, Barker & Stacey, 1979). Bag₁ fibres are larger in diameter than chain fibres in cat and rat, though they are similar in size to those of the rabbit. They are usually of smaller diameter, and shorter than bag₂ fibres in cat, though of similar length in rabbit and rat (Barker et al.,

1976a).

In the cat the M-line profile is that of a faint double line or no line in regions A and B which changes to a single M-line in region C. This change in M-line condition is accompanied by: an alteration in mitochondrial appearance, in that they change from being small and scarce to being larger and more numerous; an increase in the amount of myofibrillar sarcoplasm; and better development of the sarcotubular system. In the equatorial region the myofibrils are only present round the periphery of the fibre, the core being occupied by the bag of nuclei (Barker et al., 1976a, Banks, Harker & Stacey, 1977 , Kucera, Dovrin-Zis & Engel, 1978).

Histochemically, the alkaline ATPase profile is low in regions A and B, increasing to high in region C, whereas both phosphorylase and P.A.S. show an increase in intensity of staining going from A to B regions (Banks, Harker & Stacey, 1977, Kucera et al., 1978).

3) The bag₂ fibre

The bag₂ fibre is usually the thickest and the longest of the intrafusal fibres, and there is normally only one such fibre per spindle (Barker et al., 1976a), though exceptionally two have been reported (Banks, Barker & Stacey, 1979). Very occasionally a muscle spindle may lack the bag₂ fibre and contain only a bag₁ fibre and chain fibres (Kucera, 1982b). The polar regions of the bag₂ fibre are marked by prominent elastic fibres (Gladden, 1976). These are scarce in the poles of the bag₁ fibre and may reflect physiological differences between the two fibres.

The bag₂ fibre is the first of the intrafusal fibres to be formed (Milburn, 1973) and it associates with the chain fibres. The alkaline ATPase profile is medium, and the double M-line condition is present in the A region; the B and C regions showing

a single M-line. Both phosphorylase and P.A.S. show medium activity throughout all three regions, though there is an increase in activity in P.A.S. with distance from the equator (Barker et al., 1976a).

The intrafusal fibres are usually considered to be of the same type at both poles (Barker et al., 1976a, Banks et al., 1977b). Kucera (1981) , using a combination of myosin adenosine 5¹ triphosphatase and nicotinamide adenine dinucleotide tetrazolium reductase, has found that, although the majority of intrafusal fibres were of the same type in both poles, seven spindles contained a " mixed " nuclear bag fibre. These presented as a bag₁ in one pole and a bag₂ in the other, with differences in fibre diameter, polar length and motor innervation.

This is taken to indicate that the equatorial region can act as a boundary between the two distinctly different poles. These observations are consistent with the results of Boyd (1976a) which are that the two poles of a nuclear bag fibre often receive independent motor supplies and contract as separate functional units.

The sensory terminals

1) The primary ending

The primary sensory ending has the classical " annulospiral " appearance as described by Ruffini (1898).

The ending is usually distributed to both bag and chain fibres Banks, Barker & Stacey, 1977 & 1979), although occasionally endings occur which are restricted entirely to bag fibres (Barker & Cope, 1962).

Banks, Barker & Stacey (1982) have analysed the innervation of the intrafusal fibres by the primary^{axon}, and they found consistent differences in the form and disposition of the terminals on the two

types of bag fibre . The terminals on the bag₂ fibre were spaced widely apart and were mainly transversely-oriented, with minimal irregularity at each end, whereas, on the bag₁ fibre, the terminals were wrapped closely together and were flanked by an irregular array of terminals. Adal (1969) reports that ultrastructurally the terminals of the primary endings are similar on both bag and chain fibres, with the axons lying in shallow grooves formed by folds of sarcolemma partly overlying the axon. The basement membrane surrounding the intrafusal fibres covers the terminals of the ending (Merrillees, 1960).

It has been reported that cross-terminals can occur so that two chain fibres can be innervated by a sensory terminal (Adal, 1969 , Scalzi & Price, 1971 , 1972 , Banks et al., 1982) which may account for Boyd's observation (Boyd, 1976b) that chain fibres can act as a single unit.

2) The secondary ending

The form of the secondary sensory ending is usually that of the annulospiral ending (Barker, 1948). This is mainly distributed to the chain fibres (Boyd, 1959), and distribution to chain fibres is recognised as a constant feature of all secondary endings (Banks et al., 1982). The bag fibres can also receive innervation - Banks, Barker & Stacey (1979, 1982) report that 73% of secondaries show a bag₁, bag₂ and chain fibre distribution, and that bag₁ fibres have approximately half the area of ending as the bag₂ fibre.

A less regular " flower-spray " is another form of the ending, though less common (Barker & Ip, 1960). These spray formations have been described by Banks et al. (1982) as being formed on bag₁ fibres, and where this happens, the rest of the ending is

less regular than usual - occasionally the sprays becoming the dominant feature, giving rise to the classical " flower-spray " of Ruffini (1898).

Usually there is only a single secondary ending, though there can be up to five on any one spindle. Boyd (1962) outlines a division of secondary endings according to the distance of the ending from the primary, and terms them S_1 , S_2 and S_3 etc., according to which 400 μ m zone the ending occupies.

The afferent innervation

The primary ending is usually supplied by a single, group Ia primary afferent (diameter 12-22 μ m in the nerve trunk (Adal & Barker, 1962) which, in hindlimb muscles, does not branch between the dorsal root ganglion and the muscle spindle (von Thiel, 1959). The Ia afferent divides, usually within the periaxial space, to form two (84%), three (11%) or four (1.7%) first order branches (Banks, Barker & Stacey, 1977 , 1979 , 1982).

The distribution of these branches has been analysed, and it has been found, in tenuissimus muscles, that 73% of the first order distribution branches have a segregated_A to bag₁ and bag₂ and/or chain fibres, resulting in a separation of dynamic and static inputs. Ia axons from other muscles (e.g. superficial lumbrical) have a more mixed distribution (Banks, Barker & Stacey, 1977 , 1979 , 1982).

Secondary endings are supplied by group II afferents with a diameter lying within the range of 4-12 μ m (Hunt, 1954), though near the spindle there is an overlap with group Ia axons (Adal & Barker, 1962 , Boyd & Davey, 1968). The branching and distribution of group II axons has also been studied by Banks et al. (1982): Most II axons were found to branch to produce two (74.6 %), three (7.0 %) or four (0.7 %) first order branches.

A segregated distribution of two first order branches was found in only 22.7% of cases.

The afferent response

The physiological characteristics of these two endings have been described by Matthews (1972) and Hunt (1974).

It had been suggested that the difference between the responses of the primary and secondary endings might be due to the physical properties of the intrafusal fibres which they innervate (Matthews, 1972). However, at that stage, the distinction between bag₁ and bag₂ fibres had not been made. Now it is known that the primary ending is supplied to the bag₁, bag₂ and chain fibres (Banks et al., 1982). The position of the ending is midequatorial, which means that both the bag and chain fibres consist mainly of myonuclei for most of the length of the primary ending, while the secondary endings, which are situated juxtaequatorially, will be innervating myofibrillar regions of the intrafusal fibres. Poppele, Kennedy & Quick (1979) have shown that the stiffness of the nucleated region does not alter with changing spindle length, so that if this region of the spindle could be stretched on its own, a linear relationship between response and deformation might be expected. However, the primary response is characterised by its " dynamic sensitivity ", so the property which causes this must reside in the non-nucleated parts of the intrafusal fibres.

Poppele et al. (1979) have also shown that, on stretching a spindle, the stiffness of the polar region is initially much lower than that of the nucleated zone, but it becomes relatively stiffer as the spindle is stretched, because the sensory region is more elastic. This means that as a spindle is stretched, the rate of deformation of

the primary ending (especially over the bag₁ fibre which has more elastic fibres in the equator (Gladden, 1976)) is increased, and this could account for the non-linearity of the dynamic response. Smith (1966) and Boyd (1976b) observed the phenomenon of " creep " in " slow " intrafusal fibres after they have been passively stretched, and that such intrafusal creep is usually absent in " fast " nuclear-bag fibres. This creep can be correlated with the reduction of firing from the primary ending when the stretch is released. These " slow " fibres have been identified as bag₁ fibres (Boyd, 1976a).

The secondary ending, being more polar in its position (Barker, 1974) and being mainly distributed to chain fibres (Boyd, 1976a), innervates relatively homogeneous regions of intrafusal fibre. It has been suggested by Boyd (1981) that there is little or no functional input to secondary endings from bag fibres, and therefore a more linear response to passive stretch, from a secondary ending, would be expected, and this is seen in its static response (Matthews, 1972).

The distribution and effects of fusimotor innervation on intrafusal muscle fibres are other factors which determine the nature of the sensory response.

The transduction mechanism of the afferent response

The manner in which nerve impulses are generated from the endings and propagated into the axons is as yet unresolved. It is presumed that receptor potentials are produced by deformation of the nerve terminals, probably by squeezing the terminals between the basal lamina and the muscle fibre (Banks et al., 1982).

An electronic potential has been recorded by Hunt & Ottoson (1975), in a Ia axon, and this is interpreted by Banks et al. (1982) as a " compound receptor potential ", being produced separately by the

terminals on bag₁, bag₂ and chain fibres. It has been shown by Hunt, Wilkinson & Fukami (1978) that sodium is the main carrier of current associated with the generation of the receptor potential, but other workers (Ito, Komatsu & Kaneko, 1980) suggest that calcium ions may also play an important role in the production of the receptor potential.

Quick, Kennedy & Poppele (1980) have shown, using a ferric ferrocyanide cytochemical stain, the heminodes and some of the penultimate nodes to be potential sites of spike generation, but there is evidence to suggest that not all spikes that are generated actually propagate into the parent axon (Ito, 1969).

It has been postulated (Hulliger, Matthews & Noth, 1977) that there are two or more competitively interacting pace-makers controlling the static and dynamic components of the primary-ending response.

The outputs of the separate dynamic and static pace-makers do not summate linearly; the output of the dynamic being occluded by the static (Hulliger & Noth, 1979).

Banks et al. (1977), & Banks et al. (1982) correlate this "physiological asymmetry" with the branching of the Ia axon. They suggest that the axons associated with the static input branch more profusely compared to those associated with the dynamic, and that this would allow the static system to recover more quickly from an antidromic impulse, (from the dynamic pacemaker.) The dynamic system would recover less quickly from an antidromic impulse, (from the static pacemaker(s).)

The only satisfactory method to resolve this point would be to conduct a physiological and histological study on the same muscle-spindle.

The motor terminals

Three categories of fusimotor ending have been recognised in the spindle. These are trail endings, p_2 plates and p_1 plates.

The trail ending is almost invariably present in spindles (Barker, 1974), and it is situated in a juxtaequatorial position (Barker, Stacey & Adal, 1970). It is supplied by small diameter gamma efferents, which frequently have long pre-terminal axons. Single gamma efferents may branch within the muscle nerve and supply more than one spindle (Barker, 1968 & Barker, Emonet-Dénand, Harker, Jami & Laporte, 1976), or innervate both poles of a spindle (Barker, Stacey & Adal, 1970). One consequence of these branchings is that the trail endings on a spindle are probably innervated by several gamma axons in the nerve trunk (Barker, 1974).

The p_2 plate is also innervated by gamma efferents. It is characterised by: its knob-like axon terminals; the lack of any obvious sole plate; the relatively large diameter of the supplying axon (Barker, Stacey & Adal, 1970). It has more than twice the average length of an extrafusal or a p_1 plate. The p_2 plate is situated more distally than the trail ending (Barker et al., 1970), and the majority of p_2 axons entering a spindle terminate in one pole only, though occasionally both poles are supplied (Barker, 1974).

The p_1 plate resembles an extrafusal motor endplate, and is sited upon a nucleated sole plate, which, unlike the p_2 plate has a Doyère's eminence (Barker, Stacey & Adal, 1970). The p_1 plates are slightly longer than the extrafusal motor endplate, the average length being 33 μm (Barker, Emonet-Dénand, Laporte & Stacey, 1979), but they are considerably shorter than the p_2 plates.

Most p_1 axon branches terminate in one plate only, but branching

may occur and give rise to two or three adjacent plates on the same muscle fibre.

The exact distribution of the four types of fusimotor axon to the three types of intrafusal fibre has been the subject of some debate (Homma, 1976 , Boyd, 1981b).

The efferent innervation

The efferent supply to the spindle consists of two types of axon , those that are entirely fusimotor - gamma axons, and those which have a skeletofusimotor function - beta axons.

Functionally both the gamma axons and the beta axons can be classified according to the effect of their stimulation on the " dynamic index " of the response of a Ia afferent axon, from a spindle undergoing a ramp stretch. (The term " dynamic index " was introduced by Crow & Matthews (1964), and is defined as the difference between the firing rates at the peak of the stretch, and at half a second into the hold phase.) " Dynamic " axons increase dynamic responsiveness, whereas " static " axons depress it.

It has been found that static gamma axons terminate in a trail ending, and dynamic gamma axons terminate in a p_2 plate (Barker, Stacey & Adal, 1970; Barker, Emonet-Dénand, Laporte, Proske & Stacey, 1973 ; Barker et al., 1976 a & b). It has also been shown that beta axons terminate in p_1 plates(Barker, 1970 ; Barker, Emonet-Dénand, Laporte & Stacey, 1980).

1) The fusimotor (gamma) innervation

Studies of Barker, Emonet-Dénand, Laporte, Proske & Stacey (1973), Bessou and Pages (1975), and Brown & Butler (1973) have shown that dynamic gamma axons supply bag fibres almost exclusively, and that static gamma axons do not exclusively supply chain

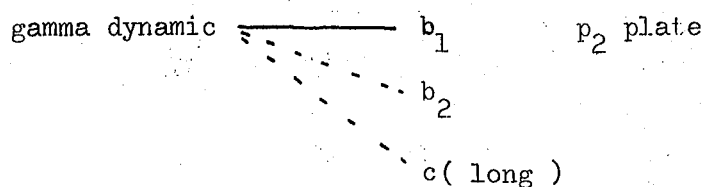
fibres. Barker et al. (1973), using silver staining following chronic degeneration of all motor innervation except single static gamma axons, showed that these axons within the range of conduction velocity $33-48 \text{ ms}^{-1}$ supplied trail endings to both bag and chain fibres.

Barker, Emonet-Dénand, Harker, Jami & Laporte (1976) showed that dynamic gamma axons depleted bag₁ fibres almost exclusively and that static gamma axons depleted both types of bag fibres and chains.

Barker, Bessou, Jankowska, Pagès & Stacey (1978) showed that out of 13 intrafusal muscle fibres supplied by static gamma axons, 8 were shown to be bag₂ and 5 chain fibres, and of 9 intrafusal fibres innervated by dynamic gamma axons, 7 were found to be bag₁ fibres, one a bag₂ fibre and one a long chain. Trail endings were supplied by static gamma axons, and p₂ plates were supplied by dynamic gamma axons.

Barker et al. (1978) conclude that dynamic gamma axons activate bag₂ or long chain fibres as well as bag₁ fibres in approximately 20% of spindles. This should produce a static modification of dynamic action and this has been observed (Emonet-Dénand, Laporte, Matthews & Petit, 1977). Boyd & Ward (1975) and Boyd, Gladden, McWilliam & Ward (1977) have, however, not seen contractions on chain fibres following dynamic stimulation, though Banks et al. (1978) showed the innervation by a single dynamic gamma axon of a bag₁, bag₂, and two chain fibres within a single spindle.

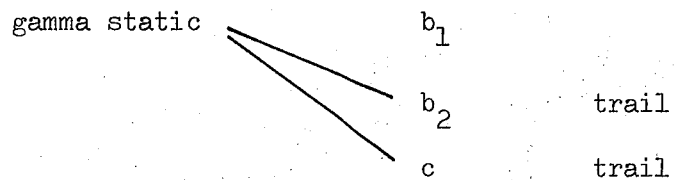
The gamma dynamic innervation can be summarised as:



The gamma static axons are shown to terminate as trail endings on bag₂ fibres and chains, but their presence on the bag₁ fibre has been the subject of recent investigation. Glycogen-depletion studies indicated that gamma static axons do make a functional innervation of bag₁ fibres (Barker et al., 1976b, Barker, Emonet-Dénand, Harker, Jami & Laporte, 1977 , Emonet-Dénand, Jami, Laporte, & Tankov, 1980), and this observation was supported by Emonet-Dénand, Laporte, Matthews & Petit,(1977). However Boyd et al. (1977a) observed no activation of the bag₁ fibre by gamma static axons, and maintained that gamma static axons only innervated the bag₂ and chain fibres.

In the recent study by Barker & Stacey (1981), where teased silver preparations were examined after chronic degeneration experiments, the contribution of gamma static axons to bag₁ fibres has been shown to be minimal (from 8% to 17%).

The gamma static innervation can be summarised as:



2) The skeletofusimotor (beta) innervation

The presence of a skeletofusimotor innervation was first physiologically demonstrated by Bessou, Emonet-Dénand & Laporte (1963 & 1965). This work was confirmed histologically by Adal & Barker (1965), who observed collateral branches of motor axons supplying both intrafusal and extrafusal muscle fibres. These beta axons were thought to terminate as p₁ plates on intrafusal fibres (Barker, Stacey & Adal, 1970). Barker et al. (1977) have shown, using the glycogen-depletion technique, that the intrafusal distrib-

ution of dynamic beta axons is almost exclusively restricted to bag₁ fibres. The zones of depletion were generally located in the mid-polar region, which is consistent with the distribution of p₁ plates.

Harker, Jami, Laporte & Petit (1977) studied the distribution of fast-conducting beta axons. They comment that, although the previous study indicated that beta axons are almost exclusively supplied to bag₁ fibres, p₁ plates are found with a "significant incidence" (Barker et al., 1970) on chain fibres. By stimulating fast-conducting axons ($> 85\text{ms}^{-1}$) selectively, then examining the whole muscle using glycogen depletion, they have shown that there are skeletofusimotor axons which contribute to the innervation of chain fibres in more than one quarter of spindles, and that it is restricted to chain fibres in 90% of spindle poles depleted. Another striking feature discovered was that beta innervation is highly specific for the long chain, when present, or for the longest chain fibre in the spindle (Harker et al., 1977; Jami et al., 1978) This is interpreted by Kucera (1982a) as reflecting the state of development of the spindle when the skeletofusimotor innervation arrives during ontogenesis. Harker et al. (1977) suggest that these fast-conducting beta axons will produce a static effect on the response of the primary ending, and this was demonstrated to be so by Jami, Murthy & Petit (1982).

Barker, Emonet-Dénand, Laporte & Stacey (1980) identified the endings of slow beta axons in spindles deprived of their gamma innervation. Using silver staining, they found that the remaining motor endings are p₁ plates, and that these were all supplied to bag₁ fibres. The distribution of the skeletofusimotor innervation can be summarised as follows:

beta dynamic	————	b_1	p_1 plates
beta static	————	b_2	
		long chains	

In summary, it is now generally accepted that the dynamic response of the primary ending is mediated by the bag_1 fibre, and it is the bag_2 fibre and the chain fibres which mediate its static response. Stimulation of fusimotor axons will alter the pattern of the response according to which intrafusal fibre they innervate.

Muscle-spindle reinnervation

Introduction

That muscle spindles can be reinnervated by sensory and motor axons after nerve injury has been established for some time: histologically by Huber (1900) and Tello (1907), and functionally by Barker & Young (1947), though after certain injuries, the gamma innervation is reported as not being restored (Takano, 1976).

Physiologically, Bessou, Laporte and Pagès (1966) have established that both afferent and efferent nerves can effect functional reinnervation, and more recently, Brown & Butler (1974 , 1975 & 1976) have shown that secondary afferents, as well as primary afferents, are restored, as are both gamma static and gamma dynamic axons.

Separate aspects of muscle-spindle denervation and reinnervation will now be discussed.

Effect of denervation and reinnervation on the spindle

One effect of denervation and reinnervation is that there is an increase in the number of intrafusal muscle fibres. Although this was not observed by Sherrington (1894) or De Reuck, van der Eeken & Roels, (1973), an increase in the number of fibres has been reported in approximately 20% of reinnervated spindles in the rat (Schröder, 1974a). In order to see if this effect is likely to be applicable to the material studied in this present work, a more thorough analysis of this and other papers will follow.

The basis of Schröder's conclusion that IMF increase is an effect of denervation, comes from observations on rat muscle spindles that have been denervated for six months, which show that the percentage of spindles possessing more than four IMF, in these muscles, has increased to over the normal level. He also reports, however, an increase in the

number of IMFs in reinnervated muscles. IMF proliferation was reported in rat muscles after various periods following the denervation operation, but the results for the various periods had been pooled. No indication is given of the period of denervation for these muscles, but the earliest recorded finding is recorded at 17 days after the operation. It follows that IMF proliferation has started by 17 days P.O. As the results for the number of IMFs per spindle have been pooled, it is not possible to tell whether reinnervation halts or reverses the proliferation process brought about by denervation. More comprehensive experiments were performed recently by Schröder, Kemme & Scholz (1979). In these, muscle spindles were analysed after three periods of denervation: 3, 6 and 12 months. The results showed a percentage increase of 7%, 51.8% and 99.3% respectively, which would imply a continual slow IMF proliferation process, proportional to the length of denervation time.

By extrapolation it can be calculated that at 17 days P.O., there would be an increase of at most 1.4% in the average number of IMFs per spindle. The figure calculated from Schröder (1974a) is 5%, i.e. three times the expected value. As this latter figure was calculated from muscle spindles which had been reinnervated, it indicates that, although IMF proliferation does occur during the denervation phase, the process may continue during the reinnervation phase. This latter observation is confirmed by the results presented by Schröder et al. (1979), where after 3 months in the reinnervation study, there is an increase of 0.9% in the mean number of IMFs per spindle (their Table 3), which is less than the combined results after 12 and 13 months reinnervation where the increase in the number of IMFs per spindle is at 31%, showing a large increase during the

time in which the spindles are reinnervated. Hence, from Schröder's work on the rat, it can be deduced that IMF proliferation occurs within 17 days of denervation and continues with reinnervation.

The increase in IMFs has also been described by Arendt & Asmussen (1976b) who attributed the phenomenon to an increase in the bag fibre population. These fibres were subsequently identified as bag₁ fibres by Kucera (1977a). They were of varying length and were situated in the poles, not usually crossing the equator. Many were identified as originating from a parent bag₁ fibre by splitting. They occurred in two thirds of muscle spindles following de-efferentation in the rat.

The fact that the complement of the intrafusal bundle is not altered numerically following chronic de-afferentation (Kucera, 1980), yet is altered following chronic de-efferentation, indicates that the proliferation of bag₁ fibres in the rat is brought about by the absence, and possibly the subsequent return, of the motor nerves only. Correlation is drawn, by means of histochemical and ultrastructural properties, by Arendt & Asmussen (1976) and Kucera (1977a) of the bag₁ fibre of the rat muscle spindle with the slow, tonic extrafusal muscle fibre of the avian anterior latissimus dorsi muscle. This muscle is known to increase its extrafusal fibre count following denervation (Sola, Christensen & Martin, 1973), so a common mechanism is implied. The mechanism by which rat IMFs increased in number following denervation and reinnervation was suggested by Schröder (1974a) to be either splitting of existing IMFs or the maturation of satellite cells.

All the recent work reported here has been performed on the rat, but it is not unreasonable to assume that similar processes may occur in the cat. The bag₁ IMF in the cat shows a slightly lower

alkaline ATPase staining intensity at all distances from the equator than in the rat (Barker, Banks, Harker, Milburn & Stacey, 1976), an observation which would not discourage the assumption that in the cat also, the bag₁ fibres will increase in number following denervation.

Although the speed with which this process occurs in the cat cannot be inferred from the rat studies, it would appear that the extent of IMF proliferation is proportional to the denervation time (Schröder et al., 1979, Kucera, 1977 and Arendt & Asmussen, 1976). If this applies in cats, the increased number of bag₁ fibres may be present at any stage following denervation. Calculations made using the figures from Schröder (1974) and Schröder et al. (1979) indicate that the IMF proliferation continues during the reinnervation stages as well; this would indicate also that increased numbers of bag₁ fibres might be present even after the shortest periods of denervation time.

Arendt & Asmussen (1976a) counted the number of spindles in rat soleus muscles following transient or permanent unilateral denervation. They claim to have found that denervation with, or without, reinnervation, reduces the number of spindles in the muscle by nearly 50%, and that this also occurs in the contralateral muscles. They reason that previous studies have used the contralateral muscles as controls, so the large alteration was not noticed. It is however surprising that the atrophied remnants of these spindles have not been seen by other workers. In fact de Reuck et al. (1973) observed that intrafusal fibres appeared to show minimal atrophic changes in size up to 4 weeks after nerve section. Arendt & Asmussen (1976a) report that, after 12 weeks of denervation, in the remaining spindles, chain fibres have atrophied slightly, though the extrafusal fibres show considerable atrophy over the same period. They also observed that bag

fibres show a small " pseudo-atrophy ", at 12 weeks, followed by a significant hypertrophy at 18 weeks, occurring in the absence of reinnervation.

An increase in the length of intrafusal fibres has also been reported by Arendt & Asmussen (1976b).

Intrafusal fibres show histochemical changes following denervation. De Reuck et al. (1973) have shown that 2 weeks after denervation, the ATPase, succinic dehydrogenase, and phosphorylase activity have started to decrease. When reinnervation began after 4 weeks they observed that the enzyme activity increased, and that extrafusal grouping of fibres with the same enzyme, took place, and they also reported that intrafusal fibres showed the same changes. Kucera however (1977b) has shown that most intrafusal fibres appear unchanged histochemically following reinnervation, though some abnormalities do occur.

Another effect of denervation on the spindles is that of thickening of the capsule. This has been described by Tower (1932 & 1939), Gutmann & Zelena (1962) and more recently Swash & Fox (1974) who claimed that such thickening is the most striking feature after longstanding denervation in man. The observation of thickened capsules was also made by Arendt & Asmussen (1976b).

The enlargement and filling of the periaxial space following denervation has been reported by Lapresle & Milhaud (1964) and Cazzato & Walton (1968), but more recently, Kucera (1980b) has reported that, following long periods of deafferentation, the capsule sheath was thicker, but the periaxial fluid space was either greatly diminished or absent. He also reported that the characteristic accumulation of fibre nuclei was no longer present in the central region of the intrafusal fibre, and concluded that the structural uniqueness

of the equatorial region depended upon the continuous presence of the sensory nerve terminals.

Restoration of terminals

Motor endplates have been described in reinnervated muscle spindles by Huber (1900) and Tello (1907), and the " spiral arrangement of rather large terminal branches " of a primary ending was observed by Huber (1900).

Ip & Vrbová (1973) claimed that following nerve crush in kittens at 2 and 15 weeks, reinnervation by motor endplates was almost complete. Their illustrations show presumed p_1 plates and presumed trail terminals, but p_2 plates are not mentioned. Sensory endings are also present, and are described as being " very different " from normal, but in some, annulo-spiral endings are just visible.

Ip, Vrbová & Westbury (1977) studied the sensory reinnervation in cats, following denervation and de-efferentation, and they reported that most of the sensory endings found in spindles, from muscles that had been denervated by crushing of the supplying nerve, had a normal appearance. After ventral root section and nerve crush however, a " variety of abnormalities " of sensory innervation was noticed. These took the form of nerves making knob-like terminals without spirals and coils, and in others, only " rudimentary contacts " could be detected. The degree of sensory restoration was correlated with the state of reinnervation of the extrafusal fibres, rather than with the time after the crush operation or with the state of the reinnervation of the intrafusal fibres. No comment was made on the form of the restored endings.

Barker & Boddy (1980) studied the spindles in cat peroneal muscles after varying periods of reinnervation , following nerve crush injury. They recorded that the sensory innervation is restored less success-

fully and more slowly than the motor; the return of beta and gamma axons preceding that of Ia and II axons. All regenerated primaries were reported as being defective to some extent, the most common abnormality being that one or other of the two types of bag fibre receives very few terminals, or even none at all. Regenerated secondary endings were recorded as being restored in their normal position on the spindle. The motor terminals, p_1 , p_2 and trail endings are all recorded as being restored, the most common abnormality being hyperinnervation. Aberrant, presumed motor axons were also observed, and it was suggested that these might be alpha motor axons which had grown down an endoneurial tube which they did not previously occupy. It was postulated that these might provide a histological basis for Brown & Butler's observation of an increased static fusimotor innervation (1976).

Physiological recovery after nerve lesions

Functional recovery of muscle spindles after nerve lesions

That muscle spindles regain function following nerve lesions has been known for some time (Barker & Young, 1947 ; Thulin, 1960 ; Bessou, Laporte & Pagès, 1966). Thulin (1960) showed that the afferent fibres to spindles were restored following nerve resection and tubulation, and these produced a sustained, low-frequency discharge to a maintained stretch, but were totally silent during a relaxed state. Gamma innervation proceeded more slowly, and, in non-tubulated nerves did not occur at all. Its presence is demonstrated by the maintenance of the afferent firing during extrafusal contraction.

1) Afferent response

a) Primary responses

Following reinnervation after nerve crush, Ip, Vrbova & Westbury (1977) found that 34% of afferent fibres, identified as originating from the primary endings of the muscle spindle, produced a response similar to normal, on stretching. Brown & Butler (1976) also reported that " many of the afferent fibres responded normally to stretching ".

The abnormalities of the response were reported as being confined to the maintained part of the discharge (Ip et al., 1977), and ranged from " an adaptation more marked than usual " to a " complete absence of maintained discharge during the phase of maintained extension."

Brown & Butler (1976) also reported the absence of a maintained response as one form of abnormality, but recorded that responses with " excessive dynamic sensitivity " were found.

b) Secondary responses

The classification of group II axons was made by Ip et al. (1977), on the basis that they could not be driven by vibration, and that they did not exhibit dynamic sensitivity. They were distinguished from tendon organs, as they had a lower threshold of discharge. Brown & Butler (1976) analysed the behaviour during muscle-twitch contractions to distinguish group II afferents from Golgi tendon organs, and then classified them as group II if they fired more regularly than group I afferents, and had low dynamic sensitivity. Ip et al. (1977) were using de-efferented muscles.

Ip et al. (1977) reported that all the responses of second-

ary endings, with a sample size of six, were indistinguishable from normal, and Brown & Butler (1976) have recorded a normally responding secondary ending in at least 35 examples.

Hyde & Scott (1983) and Scott (1982) have investigated the afferent responses of muscle spindles after varying periods of reinnervation following nerve crush. They classified the responses according to the degree of abnormality displayed. It was found that the proportion of abnormal afferents was greatest during the early periods of reinnervation, until, at 96 days post-crush, the majority of afferents (79.7%) responded normally to ramp and hold stretch.

The abnormality on which they based their classification of response was the absence or rapid failure of firing during the hold phase of the stretch. They suggest that these abnormalities may be accounted for by assuming a subtractive reduction in the firing frequency which they attribute to an increase in the pacemaker threshold.

All three groups of workers measured the conduction velocity of the axons from which they made recordings and found that the values for both primary and secondary afferents were lower than normal: the modal value for reinnervated primary afferents being 84-90 msec.⁻¹ from Brown & Butler (1976), 38 msec.⁻¹ from Ip et al. (1977) and Hyde and Scott (1983) and Scott (1982) found, on measuring the CV proximal and distal to the crush site after 33 days post crush, the CV of the distal part to be 4.5 msec.⁻¹ as compared with 41.2 msec.⁻¹ for the proximal part; by 47 days post-crush the CVs were 13.4 msec.⁻¹ and 44.2 msec.⁻¹, respectively. For secondary afferents, the values were found to be 36-42 msec.⁻¹

(Brown & Butler, 1976) and 22 msec.⁻¹ (Ip et al., 1977).

There was overlap between the two CV spectra, and Brown & Butler (1976), Ip et al. (1977) and Hyde & Scott (1983) all conclude that identification of afferent type cannot be made on the grounds of CV alone.

It is possible, therefore, that any reinnervated primary ending that had an abnormality which reduced its dynamic sensitivity, would be classified as a secondary; and similarly a group II afferent which reinnervated a spindle so as to produce an ending that had dynamic sensitivity, could be classified as supplying a primary ending. The problem of identifying the nature of reinnervating axons in terms of their normal behaviour, is one which is also present in histological studies.

Fusimotor reinnervation

Fusimotor axons have been shown to increase the afferent discharge of reinnervated spindles (Bessou, Laporte & Pagès, 1966 ; Thulin, 1960), but it is only the recent work of Brown & Butler (1974, 75 & 76) and of Hyde & Scott (1983) which has established that both static and dynamic gamma effects are also restored. Brown & Butler (1976) reported that after crush injuries, many normal static and dynamic gamma axons were found, and half of these produced an effect on more than one afferent axon (type not specified). Only one out of 56 samples produced a different effect on two different afferent axons. The conduction velocities for the gamma component were measured, and it was found that there was no shift in the diameter of the axons to being lower than normal. Hyde & Scott (1983) also identified many static and dynamic gamma axons, and concurred with Brown & Butler that regenerated gamma axons which had their actions confirmed on more than one afferent

ending, were invariably consistent in their effect.

Skeletofusimotor axons have been identified which produced either static or dynamic actions, and occasionally both, on spindle afferents (Brown & Butler, 1976). Six of the beta axons acted on more than one unit, and were consistent in their action. In all, 18 examples of beta axons were found in crush and 23 in cut, and they claimed that this showed beta axons to be " much more common " in cut than in crush.

Nature of lesion and differential rates of return

Comment was made even during the very early studies, that there might be differences between the rates of reinnervation of sensory and motor axons. Huber (1900) observed that, under " favourable conditions " following crush of the posterior tibial nerve in the rabbit, the regeneration of nerve terminals in the " complicated neuromuscular end-organs " might be complete, and described the spiral arrangement of large terminal branches of axons traced to neuromuscular spindles, and concluded that motor nerve endings regenerated more quickly than sensory nerve endings.

Tello (1907), in a study of reinnervation following section of the sciatic nerve in rabbits, did not make any conclusion as to the relative rates of reinnervation of sensory and motor axons to the " spindles of Kühne ", but he illustrated the process with a drawing, showing the motor arborisation of a spindle to be well-developed, while the sensory branches were still growing under the capsule.

More recently, Ip & Vrbová (1973) studied the reinnervation of spindles in kittens. After crushing the sciatic nerve in two kittens, one at 2 and one at 15 weeks, they showed that the motor reinnervation of intrafusal muscle fibres was almost complete, but that the appearance of sensory endings was abnormal. They also concluded that the

sensory reinnervation proceeded more slowly than the motor, and that this effect was more pronounced in young animals.

In the combined electrophysiological and histological study published by Ip et al. (1977), it was observed that the establishment of normal afferent terminals was dependent, to some extent, upon the efferent reinnervation.

The observation that motor axons return faster than sensory axons, after crush lesion, was confirmed by Brown & Butler (1976), who were invariably able to record fusimotor activity from afferents, during the early stages of reinnervation.

The three studies of Huber (1900), Ip & Vrbová (1973) and Brown & Butler (1976) all indicate that, following nerve crush, the motor reinnervation of spindles proceeds faster than the sensory.

Cut lesions provide a more difficult obstacle to reinnervation; Barker & Young (1947) commented that some spindles might never be reinnervated after nerve section. Brown & Butler (1976) also recorded that recovery was more rapid and complete for both afferent and efferent axons after crush than after section.

Thulin (1960) resectioned the nerve and found that, in some cases, gamma axons were never restored to spindles, and even when the cut ends of the nerve were tubulated, gamma axons were much later in returning than the group Ia afferents. Homma (1969) also reported that, following cut and suture of the nerve, the gamma component is slower in returning than the group Ia afferents. The results from both these studies indicated that this could be a function of the diameter of the axons concerned, as alpha motoneurons and group Ia axons were restored at similar times in both cases, and group II afferents were found to return later, with the gamma component (Homma, 1969).

Nerve freeze has also been used to produce denervation and reinnervation of spindles. Takano (1976) reported that, even after 6 months, there was a total absence of gamma innervation, though functional afferents were present from the end of the fourth month. De Santis (1972) reported reinnervation, after nerve freeze, by afferents, but motor innervation was not discussed.

The presence of aberrant motor axons

The first indication that muscle spindles might be reinnervated by aberrant motor axons was given by Tello (1907). He suggested that the presence of these axons, in the spindle, might be caused by the regenerating motor axons entering an endoneurial tube which had previously contained a sensory axon.

More recent workers have produced a similar explanation for some of their observations. Schröder (1974) suggested that erroneous alpha or beta innervation of intrafusal muscle fibres, by the aberrant regeneration of nerve fibres, normally innervating extrafusal fibres, could be the cause of localised hypertrophy in rat muscle, after reinnervation following nerve crush.

Histochemical abnormalities in reinnervated spindles have also been explained by the postulated existence of erroneous connections from inappropriate motor axons (Kucera, 1977b). Physiological evidence for such axons is provided by Brown & Butler (1976), who interpreted their observation that beta axons were not found as commonly after crush as after section, as implying that many of the beta axons arose as a result of injury; i.e. the spindle is being reinnervated by axons which, prior to the lesion, terminated entirely on extrafusal fibres. Histological evidence to support this suggestion has been provided by Barker & Boddy (1980).

Specificity of reinnervation

Morphological studies on reinnervated spindles have revealed that, after nerve crush, regenerated sensory nerve terminals are only found in the equatorial region, and that motor nerve terminals are only found in the poles (Schröder, 1974). De Santis et al. (1972) also report that no motor terminals are found in the equatorial region of muscle spindles following nerve freeze. These observations indicate that the reinnervation of spindles is specific in so far as sensory terminals and motor terminals are confined to the general areas that they occupy in the normal spindle.

The fact that histochemical profiles of reinnervated intrafusal fibres nearly always resume their normal appearance (Kucera, 1977a) could be explained by the existence of a highly specific reinnervation process, but a high resistance to metabolic conversion may also provide an explanation for this observation.

Brown & Butler (1976) argue in favour of specificity of reinnervation; the strongest evidence being that when gamma axons can elicit an alteration in response on more than ^{one} afferent, it is almost invariably the same alteration that occurs in both cases, i.e. both are dynamic or both are static. Brown & Butler (1976) maintain that this observation, together with the fact that the characteristic response patterns of reinnervated primary and secondary endings were usually associated with axons whose conduction velocities were close to those found in normal spindles in cats, indicates that the reinnervation of spindles is a specific process. There is, however, little evidence from the morphological studies, as to how precise this specificity is.

Concluding Remarks

It is intended to investigate the reinnervation process using a whole-muscle staining technique. This would allow the distribution of nerve terminals to be assessed over both the intrafusal and extrafusal fibres.

Previous work has indicated that motor axons return faster than sensory axons after nerve crush, and this has led to claims that restoration of the primary ending is held back, in the absence of efferent innervation.

There is also strong evidence that the nature of the lesion has a marked effect on the success of reinnervation. By studying spindles at regular intervals after the different nerve lesions, it is hoped to investigate these effects.

There are observations in the literature which have been interpreted as indicating the innervation of the intrafusal bundle by previously exclusively extrafusal motor axons. It is hoped that, by using whole-mount preparations, the existence and identity of such axons may be assessed. It is also possible that, by using this technique, effects of denervation on spindles, such as the suggested splitting of bag fibres, may be observed in relation to the innervation.

It is anticipated that, by being able to study the distribution of the primary terminals to the intrafusal fibres, an explanation may be found for the abnormalities in response found by Ip et al. (1977) and Brown & Butler (1976).

Another reason for this study is to investigate the specificity of reinnervation of spindles, which has been suggested to be highly specific, but there is little histological evidence in support of the suggestion.

Before this study could proceed, a satisfactory staining method had to be devised.

CHAPTER TWO

CHAPTER TWO

STAINING TECHNIQUE

In a study the main purpose of which is to provide an overview of muscle-receptor reinnervation, the histological technique adopted would ideally furnish large numbers of muscle spindles and tendon organs with their surrounding extrafusal fibres, so that the course of reinnervating axons could be traced intramuscularly.

Techniques using thin sections and reconstruction have two main drawbacks: firstly they are extremely laborious, and therefore would not provide an adequate sample of spindles, and secondly, tracing very fine axons through consecutive sections would pose considerable difficulties.

Methods which employ thick sections or slices of muscle are more feasible, but to be of use, the entire spindle, and any axons associated with it, have to be within the plane of the section, a condition which would only be obtained rarely.

The more satisfactory methods of whole-muscle staining and subsequent removal of spindles and tendon organs by teasing, would allow a more thorough analysis to be made.

The established methods which may be applied to whole-muscle staining include Gold Chloride. Methylene Blue, and the silver methods. Other possibilities for this application are the mitochondrial stain used by Ovalle (1971) and those using H.R.P. techniques.

The Gold Chloride method of Gairns (1930) has been used successfully for some years. However, compared to preparations obtained with silver techniques, they have a very coarse appearance. Reinnervated material was expected to display many very fine axons, and a granular stain would not be satisfactory for these.

Ovalle's stain for sensory terminals relies on the presence of mitochondria within these terminals. This condition may still apply in newly reinnervated material, but its use in this study is precluded by the fact that the motor innervation is not adequately differentiated with this stain.

H.R.P. techniques may prove ideal for the staining of nerve terminals of spindles, however problems were anticipated, in that injection into muscle would probably obscure many intrafusal endings, and the application of H.R.P. to a regenerating nerve trunk may produce equivocal results.

Methylene Blue can produce well-stained preparations, but a pilot study indicated that a considerable investment of time would be required, both before its adoption and during its use.

As most of the histological studies of muscle spindles have been performed using silver-stained preparations, it was decided to use one of these.

The modified De Castro method of Barker & Ip (1963) has produced many well-stained spindles, but, prior to this study, the failure rate of staining, even of the motor component, was unacceptably high; in fact hardly any adequately-stained spindles were being produced .

A consultation with Ip (personal communication) revealed that he had on average a 50% success rate, using Barker & Ip's modification. This was considerably higher than was being achieved, though even this level was not satisfactory in a regeneration study, where the number of animals per time period was strictly limited.

It was therefore decided that it was essential to devise a silver-staining protocol which could be relied upon to supply sufficient well-stained spindles and tendon organs.

To this end it is pertinent to review the theories behind silver-staining techniques.

1) Theoretical basis of silver staining

In chemical terms, there are three kinds of silver impregnation (Disbrey & Rack, 1970). In the first kind, used in the von Kossa method for calcium salts (1901), the silver salt reacts with a tissue component to produce an insoluble silver salt, which is then reduced to metallic silver by strong light. The second kind of silver stain relies entirely on the strong reducing properties of the tissue, as in the Masson-Fontana method for melanin (1914).

The third kind, of which the neurohistological methods are a part, produces a deposit of metallic silver with the help of a reducing agent, and it is the mechanism of these methods which will be discussed.

a) Silver neurohistological methods

Since their inception by Krause (1843) silver stains have been notorious for their caprice, and, as a result, have been the subject of numerous modifications, and several papers reviewing the theory of the mechanism of their operation.

The early methods of Cajal and Golgi (1913 , 1879) were developed for use in whole tissue, but subsequently most work in this field has been concentrated on developing techniques for use on sections. The problems involved in perfecting a technique for sections are considerably less than those involved in producing a uniform stain within a whole muscle, because the thickness of the muscle must necessarily mean that the solution used in the procedures cannot reach its centre under

the same conditions as those at the periphery. However a review of the work performed on the mechanism of silver staining on sections is relevant to the investigation of a whole-muscle staining technique.

It was suggested even by early workers (Liesegang, 1911) that successful silver staining relies on the formation of silver nuclei. These are said to be formed during impregnation, by reducing elements within the tissue, and on development, the remaining reducible silver is deposited around these nuclei making structures visible.

This idea was disputed by Cajal (1921, reported by Zon, 1939) who claimed that treating tissues with oxidising agents that would react with such reducing elements, did not alter the nature of the staining, but it has since been shown that argyrophilic substances in nerve fibres are blocked by strong oxidising agents (Palmgren, 1948).

That tissues are capable of reducing silver solutions has been confirmed by Samuel in two ways (1953b). Firstly tissue elements were visible, due to deposited silver after prolonged incubation, but without development; and secondly he showed the presence of reduced silver in an impregnated section, by removing unreduced silver through washing with a sodium sulphite solution, and subsequently using a physical developer, of the kind suggested by Pearson & O'Neill (1946) , to produce differential staining.

Other workers have disputed the importance of such silver nuclei. Zon (1939), although acknowledging the presence of reducing substances in tissues that might form silver nuclei,

concludes that they are only of secondary importance, as they must be highly protected by colloidal gels. This observation itself is questioned by Wolman (1955a&b), who maintains that fixed tissue cannot display colloidal properties.

Silver (1942a) moreover argues that there is no specific chemical reaction between silver ions and tissue elements. He supports the hypothesis that the specificity of silver staining lies entirely in the development stage, but experiments by Samuel (1953) have shown that the conditions of impregnation do play an important role in determining specificity.

Holmes (1943) claimed that nuclei of reduced silver are formed in impregnated sections of formal-fixed material, but Peters (1955a) performed similar experiments and suggests that the method employed by Holmes gave no true indication of the presence of silver nuclei.

Peters (1955a), however, reports subsequent experiments in which sections were impregnated, then washed and treated with a physical developer, and he found a complete development of stain, confirming similar observations by Samuel (1953a).

Peters continued the experiments by placing non-impregnated sections into the same physical developer and these did not show any specific staining.

To recapitulate: the consensus of opinion from the experimental studies of Samuel (1953a&b), Holmes (1943), Romanes (1950) and Peters (1955a-e) is that nuclei of reduced silver play an essential role in the differential staining of tissues. However, the existence of silver nuclei can only be assumed from indirect evidence, as the size needed for a centre of

deposition has been shown , from studies of the photographic process, to be only a few atoms of silver.

There is another school of thought which suggests that either silver nuclei are not formed (Silver, 1942), or they are formed, but are not relevant to differential staining (Zon, 1939). It may be possible that different silver stains make use of these different effects. It would of course be relevant to establish whether the modified De Castro method relies on the differential distribution of silver nuclei or not.

If the differential nature of the stain is determined by the nuclei of reduced silver, then it is important to know the factors which affect their formation.

Using a physical developer and radioactive tracing, it has been shown by Peters (1955c) that the rate of formation increases with temperature, silver ion concentration and time, and that once formed, they are very stable. He also showed that the best differentiation of nerve fibres occurred when the pH of the impregnating solution was 8, and that when pre-treated with blocking agents, impregnated at low pH or impregnated for long periods of time, the nuclei did not give a specific stain on development.

The mechanism of formation of nuclei was also investigated by Peters , but results were inconclusive. He suggests (1955a) that if silver nuclei are formed by chemical reduction, then aldehyde groups might be responsible, whereas, if nucleus formation were by physical reduction, then silver would be reduced by virtue of the redox potential existing in the tissue. Experiments conducted by him (1955a) with blocking agents, which

attack different end-groups of amino acids, suggest a physical reduction, as they produce an indiscriminate, but similar effect.

Palmgren (1948), Holmes (1943) and Rowe & Hill (1948) agree that the selectivity of the stain depends on the fixative employed, which is interpreted by Peters (1955a) as evidence for physical reduction.

Fixatives that increase the reducing power of tissues are formalin, chloral hydrate, pyridine and ammoniacal alcohol, whereas ethyl alcohol alone decreases it (Palmgren, 1948).

i) " Reducible silver "

As well as forming nuclei within sections, it was hypothesised by Liesegang (1911) that silver ions react with tissue proteins, and that it is these which are reduced by the developer. This fraction of the silver is termed the " reducible silver ".

Samuel (1953a) reasons that on development, the pH of the reducing solution affects both the silver-holding capacity of the section and the reduction potential of the reducer. As the developer penetrates the section, there must be a fluctuation in pH associated with variable diffusion currents and Samuel therefore concludes that the use of reducible silver is an uncontrollable process. He advocates a method which relies on physical development around silver nuclei alone, after the removal of the non-reducible silver. He does, however, report that the quantity of reducible silver depends on the properties of the sections, the pH of the impregnating solution, and on the time and temperature of impregnation.

Peters (1955c) performed more comprehensive experiments to study the impregnation process, and found that there was a rapid increase in staining up to pH 9 and little staining below pH 7 when the sections are placed in a weak reducer. He suggests that, as strong reducers can produce reduction when impregnation is below pH 7, the combination of silver within the section involves some weak group rather than hydroxyl groups. However, during these experiments, he uses intensity of staining as a criterion of the amount of silver present, and, as this has been shown to depend on the distribution of silver nuclei, his results and observations cannot be solely attributable to the " reducible " fraction of the silver, but rather to the combined effects of " reducible " and " reduced " silver.

By using acidified hydrogen sulphide solution ,to precipitate silver sulphide in situ, it has been shown that the amount of silver combined with a section increases with the concentration of the impregnating solution (Peters, 1955c). This suggests an equilibrium, and therefore that the combination of " reducible " silver with the section is reversible, and that it is probably due to a weak grouping of silver with proteins.

By blocking protein end-groups, Peters attempted (1955a) to show which amino acids were involved. The results indicated that carbonyl groups may play a part in determining the specificity of the stain, but that they are not responsible for the general non-specific uptake of silver, which probably combines with the imidazole group of histidine.

Using radioactive silver Ag¹¹¹, Peters (1955c) showed the effects of pH, time, silver concentration and temperature of

impregnation, to be qualitatively similar to those for the formation of silver nuclei. However, the hydrogen sulphide experiment demonstrated that the reducible fraction of silver is distributed throughout a section, and therefore differentiation of the stain from this fraction only takes place at the development phase.

b) Development

It was considered by Liesegang (1911) that the characteristics and the distribution of the silver nuclei determine the final specificity of the stain. Although it has been demonstrated that the correct distribution of silver nuclei is essential for differential staining, the final result must depend on the action of the reducing agent.

By examining the reduction of silver nitrate solutions under a microscope using various reducers, the nature of precipitation was examined (Palmgren, 1948). Hydroquinone or metol produce a uniformly distributed granular precipitate which is deposited as the reducer spreads by diffusion. Pyrogallol also forms granulated precipitates at first, but then grows in the form of coiling threads, as silver ions are evidently adsorbed and reduced at the ends of these threads. Palmgren (1948) surmised that this difference in the mode of precipitation may be due to the speed at which the two reductions take place. He suggests that, as nuclei are deposited throughout sections and only preferentially on nerves, then selective staining can only take place if the potential stain of the reducible silver were removed from other structures. His observations that an instantaneous reduction by hydroquinone, metol or pyrogallol results in

a uniform staining of all tissue structures, corroborate this suggestion, and lead to a theory of deposition as follows. On treatment with a weak reducer, " reducible " silver is deposited around silver nuclei. These are more concentrated in nervous tissue, and, as the process is autocatalytic, the difference between the deposition rate in non-nervous and nervous tissue will become more marked. This will cause the diffusion of reducible silver from the non-neural tissue to the site of high deposition rate within the nerves. This process would enhance the contrast between the nerves and other tissues.

Using various reducers, Palmgren (1948) found that, within certain limits, the selectivity was directly proportional to the delay in the reduction. If the reduction is too slow, however, then the reducible^{Ag} will diffuse out into the reducing solution before deposition can take place, so a balance has to be struck between the speed of reduction and the loss of reducible^{Ag} by diffusion.

A different view of the reduction process was held by Silver (1942) who explained the process of silver deposition in terms of the colloidal properties of the silver solution. She claimed that negatively charged micelles of silver are formed within the solution, and that these micelles are caused to precipitate by oppositely charged surfaces. Using electrophoresis, Silver showed that silver micelles are invariably negatively charged, different pHs making them more or less negative.

The charges on tissues will vary in their intensity according to the iso-electric points of the constituent proteins, and, by

adjusting the pH at reduction, different tissues may be preferentially stained.

Samuel (1953a) discussed Silver's hypothesis and reasoned that, if it were true, then sections impregnated at different pH levels, and rinsed and dried in an incubator before development, should exhibit staining determined only by the pH and the properties of the developer. He showed that this was not the case, as there was a marked difference in staining between sections impregnated at pH 7.4, and pH 8.8, even though they had been washed and dried.

Zon (1939) discussed the physical chemistry of silver staining. He considered that an essential feature of the staining process is the deposition of silver. He favoured the theory that deposition of silver is controlled by the protective action of the colloids within the tissue. That is, that tissues which offer a high degree of protection to silver ions combined with it, will only be reduced by a strong reducing agent, whereas tissues which offer only a low degree of protection will be reduced by weak reducing agents. He suggests that the fixation process is probably the most important variable in silver staining, as it can change the protective power of the gels and also affect the ability of the section to adsorb silver. But as was previously reported, Wolman (1955) suggests that the proteins of fixed tissues are denatured and cannot be considered to have colloidal properties.

c) Summary

Silver staining depends on the successful differential distribution of nuclei formed when silver solutions diffuse into tissues. They are probably formed by physical reduction, and the

factors which affect their formation are: the fixative; the concentration, temperature and pH of the impregnating solution; and the time spent in the impregnating bath.

Silver also reacts to form an unreduced fraction which is also affected by the same factors, and which is probably reacting with histidine in the tissue proteins. The distribution of this reducible^{silver} is non-selective, and the selectivity is governed by the reduction.

If reduction occurs too quickly, then the silver deposits in situ; if the reduction occurs more slowly, then the staining is selective, but the slow speed must be balanced against the loss of reducible^{Ag} by diffusion.

As the modified De Castro method of Barker & Ip has produced adequately stained spindles in the past, this method will be examined in the light of the theoretical work previously discussed, in order to examine how it may work and why it was not working. Passages in inverted commas are quoted verbatim from Barker & Ip (1963).

Fixation:

- (i) " Fix muscle for 4-6 days in freshly prepared chloral hydrate, 1g; 95% alcohol, 45 ml.; distilled water, 50 ml.; conc. nitric acid, 1 ml. "

The use of hypnotics in fixatives (chloral hydrate) was considered by De Castro to be efficacious in the staining of nervous tissue, and it has subsequently been shown to increase the reducing power of neuronal elements. Alcohol has been shown to lessen the reducing power, but as the method has worked previously, this is not sufficient to negate the effects of the chloral hydrate. Nitric acid was

probably added to assist in the softening of the muscle. However, the pH of the fixative is also likely to alter the effect of the chloral hydrate and alcohol on the proteins. The measurement and content of 1 ml. of nitric acid could lead to large deviations in the pH of the fixative solution. This could be a source of unreliability of staining.

- (ii) " Wash for 24 hours in running tap water."

This stage is presumably intended to remove unreacted components of the fixative. Tap water is, however, in itself of varying pH and ion content. These could interact with the tissue and be another source of unreliability of staining.

- (iii) " Place for 24-48 hours in 95% alcohol, 25 ml.; ammonia, 1 drop"

Ammoniacal alcohol has been shown to increase the reducing power. The addition of 1 drop of ammonia (880) is again potentially extremely variable in the effect it would have upon the pH of the muscle, and would therefore affect the deposition of silver nuclei (and reducible silver in the next stage.)

- (iv) " Blot the surplus fluid, and incubate for 5 days in 1.5% silver nitrate, at 37° C. "

A conventional ^{on} impregnation, with presumably sufficient time for adequate formation of silver nuclei and reducible silver.

- (v) " Reduce for 2 days in freshly prepared hydroquinone, 2g; 25% formic acid, 100 ml. "

The modification of substituting formic acid for formaldehyde in the reducer was introduced in order to obtain whole preparations, because it was thought that the formic acid made teasing easier.

This was a most unusual course of action, as it had been

previously by Holmes (1943) that hydroquinone is only active in alkaline solution. To add a chemical which nullifies the effect of the reducer, in order to make the teasing process easier, could not be predicted to produce a viable stain. In fact, Barker & Ip probably chanced upon the effect that allowed their modification to work. The presence of formic acid may indeed make the muscle easier to tease, but its significance is far outweighed by its effect upon the speed of reduction. This will be discussed in detail later.

(vi) " Rinse in distilled water; clear and store in glycerine before teasing. "

(vii) " Mount teased preparation in polyvinyl lactophenol; if mounted in glycerine, ring coverslip immediately with pitch."

It was apparent that in at least four stages in the Barker & Ip modification, uncontrolled variables were present which could seriously alter the efficacy of the stain. As the inherent variability within the method was so large, it was decided to standardise it before any investigations were made.

2) Standardisation procedure

It was inevitable at this stage that the conditions chosen would be somewhat arbitrary, but they were matched to the conditions used in the Barker & Ip technique (1963).

a) Dissection procedure

Prior to this work it had been found that tissue removed from animals which had been dead sometimes for many hours, had produced some very fine preparations.

A time of $\frac{3}{4}$ hr. post mortem before dissection was adopted. The animals were killed with an overdose of Sagatal, injected intraperitoneally.

b) Position of muscle within solution

It was decided that each muscle should be suspended in solution to allow uniform penetration of reagents. The arrangement eventually adopted is shown in Fig. 2.1.

The muscle is suspended by a fine nylon thread from a polystyrene label on which identification can be scratched. The label is then hooked into a slot in a polypropylene ring. The polypropylene ring fits tightly into the neck of a screwtop glass jar, and allows for the top to be positioned, reducing evaporation and the possibility of contamination.

c) Ratio of volume of muscles to volume of solutions

By using identical screwtop glass jars, and the same set of muscles from cats of similar weights, a relatively constant ratio was obtained.

d) Glassware

Each jar contained only one kind of solution, to prevent the possibility of contamination, and was thoroughly cleaned after each usage.

The impregnation stage was performed in one of the usual glass jars, but painted matt black on the outside, as this was considered to be the only photosensitive stage.

Transfer of muscles between bottles was achieved by moving the ring from which the muscles were suspended.

During all stages except washing and impregnation, the bottles were placed in shaking water baths at 18°C.

Table 2.1

Analysis of water supplies to Durham.

	OLD	NEW		
Fluoride	0.1 mg/l	0.6 mg/l	x6	up
Aluminium	1.0 mg/l	0.01mg/l	x100	down
Chloride	7.6 mg/l	15.7mg/l	x2	up
Manganese	0.1 mg/l	0.01mg/l	x10	down
Zinc	0.05mg/l	0.1 mg/l	x2	up
Hardness	25mg/l	65 mg/l	x3	up
Alkalinity	23 mg/l	23 mg/l		no change
Sulphate	23 mg/l	30 mg/l	x $\frac{1}{2}$	up
pH	9.1	7.2		

For the silver impregnation stage, the bottles were placed in a shaking water bath, in the dark, at 37 C.

The " washing " stage presented a problem over standardisation. The method as practised used tap water, cleaned by passing it through a photographic filter, into a beaker in which the muscles were lying. This had many inherent sources of variation: the rate of flow of water; the chemical content of the water; the uneven passage of water over the muscles.

To eliminate the first two variables, it was decided to use a 30 litre aspirator to store the washing water, which could then be finely controlled as to its chemical content and flow rate.

The muscles were suspended in a large jar (Fig. 2.2.) which was itself placed in a sink of running cold water. This was to keep the temperature of the jar down, as in the preliminary runs, it had been noticed that muscles became too friable if they were allowed to warm up to room temperature at this stage.

The obvious candidate for a standard washing solution was distilled water. This was tried for several runs, but without success. Enquiries were made to the Water Board, and it transpired that the water supply to Durham had changed since the late 1960s when this silver method was known to work more successfully than today. An analysis of both past and present water supplies was requested (Table 2.1). Water was obtained from the previous source and used for two runs. There was an improvement in staining. Using this water was however not a practical proposition, as it had to be collected some distance from Durham, would still suffer fluctuations in content, and could not be repeated elsewhere.

A comparison of the two analyses was made, and the most striking differences were the aluminium ion content and the pH of the water (Table 2.1).

To determine if either or both of these factors had any effect on staining, three kinds of " artificial tap water were tried:-

- 1) Solution of Aluminium Sulphate. 1gm/5 litres
- 2) Distilled water made to a pH of 9.4 with saturated NaOH.
- 3) Solution of Aluminium Sulphate made to a pH of 9.4 with saturated NaOH.

Of the three, Aluminium Sulphate made to pH 9.4 produced the best results, and this was adopted as the standard " washing " solution.

The standardised chemical concentrations are based on Barker & Ip's method as used in this laboratory. These and the procedure for the standardised method are shown in the Appendix.

3) Investigations

Once the method had been standardised, it was possible to investigate the individual steps in the staining process. It was decided to start at the reduction stage and work backwards through the method.

Previous workers with silver stains have used three basic methods of reduction. Samuel (1953) advocates the removal of reducible silver, before a controlled physical development. This he claims would make the reduction less subject to the variable diffusion currents affecting the pH as the reducer penetrates. Though this may work well on sections, whole muscle would not lend itself to this approach, as removal of the reducible silver would take many

days of washing, and the physical developer would cause a very steep gradient in the density of the staining between the centre and the periphery of the muscle, and this would limit the number of adequately-stained spindles.

Another method of reduction uses a mixture of a silver solution and a reducing agent without the removal of the reducible silver e.g. that of Pearson & O'Neill (1946), whereas other methods of silver staining rely on the presence of reducible silver alone, which is then deposited around the silver nuclei formed during the impregnation; the Barker & Ip method is one of these (1963).

As it is unlikely that there would be insufficient reducible silver within a muscle that had been soaked in 1.5% AgNO_3 for 5 days, it was decided to maintain the method as one using reducible silver. The first investigation was into the effect of different lengths of time within the reducer, without changing the nature of the reducing solution. The times chosen were 1, 6, 12, 24, 36 and 48 hours. The shorter periods of reduction produced extremely dark, opaque muscle fibres which were difficult to tease, and no spindles were found. For the time periods between 1 and 2 days, the extra-fusal and intrafusal fibres had assumed the red and golden colours which are present in well-stained preparations. After time periods longer than this, the muscle fibres became more straw-coloured. Nervous tissue did not stain satisfactorily during these runs, but, using the colour of the muscle as an indicator, it was decided that the optimum time was two days.

As mentioned previously, the use of hydroquinone with an acid is an unusual combination as a reducer. An investigation was therefore instigated into the effects of the components of this reducer and their interactions.

The application of hydroquinone alone produced extremely dark, opaque muscle fibres which were very difficult to tease. Hydroquinone followed by formic acid also produced extremely dark muscles which were very resistant to lightening by further immersion in formic acid. It was suggested by Palmgren (1948) that the contrast between different tissues in silver staining, comes about by a process which produces the Kotinsky effect in photography (Mitchell, 1948). That is, the reduction takes place sufficiently slowly , so that areas of high silver nuclei density draw silver ions from surrounding low density tissues by diffusion down a concentration gradient, as silver ions are being removed by reduction more quickly in the regions of high silver nuclei density.

The results therefore indicate that hydroquinone alone was too powerful as a reducing agent, and did not allow adequate differentiation to take place. Formic acid by itself has the effect of clearing the muscle, and removing excess stain, but does not cause differentiation.

When used in a mixture with hydroquinone, formic acid lessens, but does not abolish its power as a reducing agent, allowing differentiation to take place. As speed of reduction is an important factor in causing differentiation, a series of experiments, using the standard concentration of hydroquinone in different concentrations of formic acid, were performed.

The concentrations used were 1, 5, 10, 20, and 25%. None of the concentrations gave a better result than the 25%, the standardised concentration.

Another reducing combination which has been used successfully is a hydroquinone/ sodium sulphite mixture (Holmes, 1943). This

was tried in place of the standard reducer, using 12, 24 and 48 hours of reduction; none produced an improvement in the result.

Other reducers were tried, for example formalin and pyrogallol, but there was no alteration in the success of the staining.

It was concluded from these experiments that the lack of success in the stain was not caused by a fault in the reducing process, and that the most satisfactory reducer for this staining method is hydroquinone in a solution of formic acid.

It had been observed during these experiments that areas of teased muscle were stained differently in terms of intensity and colours to others, and that well-stained spindles only came from within one type of extrafusar staining. When a stained muscle was sectioned transversely, it could be seen that these differently stained areas were formed by concentric rings of staining within the muscle, with the well-stained spindles being confined to a relatively narrow, darkly-stained ring. This differential staining could be due to the effect of the reducer diffusing only a small distance into the muscle. To test this hypothesis, a muscle was sectioned transversely after impregnation, but before reduction, and the cut face was exposed to the reducing solution. If no rings were formed on the cut face, then it could be inferred that their formation was due to diffusion of the reducer. However, the usual pattern of rings was present on this cut face. This implies that the selectivity of staining, which causes ring formation, is determined by a process prior to the reduction procedure, and thereby contradicts the theories of Zon and Silver (1939 & 1942).

Another factor which may be responsible for the formation of these rings is the diffusion of the fixative into the muscle. In order to test this possibility, a similar experiment to the previous one was performed. In this experiment, a muscle was sectioned transversely after its immersion in fixative, and then processed as usual with the cut face exposed to all solutions. Even after the removal of superficial silver deposits, no rings were visible. This implies that the critical stage in the formation of these rings (and presumably thereby, the correct conditions for good staining of muscle spindles), was during the penetration of the impregnating silver solution.

It had also been found that the staining of axons and terminals within the capsule of the spindle was frequently very poor, even though the axons in the polar region were well-stained.

An hypothesis was devised to explain these two observations. It assumes that the differential axonal staining : ~~now~~ depends on the pH as the impregnating solution reacts with the tissue; the validity of this assumption being suggested by the previous work on sections (see Chapter Two - Theoretical basis of silver staining).

Prior to their immersion in the silver solution, the muscles have been in ammoniacal alcohol at pH 9. On immersion in the impregnating solution, the sequence of events in the muscle can be represented as in Fig. 2.3a . That is that at some stage, every point within the muscle passes through all pH values between 9 and 6. As the quality of staining is not uniform throughout the muscle, another factor must be acting in conjunction with this. The most obvious candidate for this is the silver ion concentration. This would follow a progression illustrated as follows:- (Fig. 2.3b)

If it were assumed that for a correct deposition of silver nuclei and reducible silver, that the pH has to be at 7.5, while the concentration of silver remains at 0.75% , then the only position on the radius of the muscle at which these conditions obtain at the same time, is at D, which could be seen as a circle on a transverse section.

The lack of staining within the spindle capsule would be explained if the capsule were considered to be a diffusion barrier. This would mean that conditions within the capsule were lagging behind extracapsular conditions, and frequently not reaching the correct conditions for adequate staining. Such a hypothesis would also account for the presence of the occasional well-stained capsular region within a totally overstained muscle.

There are several possible approaches to solving the problem of the diffusion of silver solutions.

If the distance through which the silver solutions had to diffuse were very short, e.g. less than 2mm., then conditions of impregnation could be under tighter control. This could be achieved by using thick sections, or thin muscles. The former option had been rejected already for reasons outlined previously, and it had already been decided to use the peroneal muscles. Another option was to squash the peroneal muscles. Despite injections with hyaluronidase and sonication, muscles could not be relied upon to maintain a flattened form.

Another technique would be to control the pH of the impregnating solution by the use of buffers. Experiments were performed using silver solutions of varying pH, from 7 to 9, buffered using the borax/boric acid mixture suggested by Holmes (1948), at diff-

erent concentrations of silver, but no examples of uniform staining were found. Other experiments employing ammoniacal silver solutions also failed to produce a uniform stain. It was therefore decided to adopt an alternative approach.

If a diffusion barrier were placed around a muscle, then the theoretical sequence of events shown in Fig. 2.4 could occur. Such a barrier could have the effect of making conditions more even throughout, and a larger volume of well-stained material would be present throughout the whole muscle. This might also improve the staining intracapsularly, as conditions inside the capsule might be able to "keep up" with those which change more slowly in the extracapsular region.

In order to test this hypothesis, three diffusion barriers were applied before immersion into the impregnating solution. These were Collodion, Neobecutane, and 1% Agar gel.

The reduction process, though necessarily slow, in order to produce differentiation, needs to progress as evenly as possible throughout the muscle. The diffusion barriers were therefore removed from the muscles prior to their immersion in the reducing solution.

All three diffusion barriers produced muscles which yielded large numbers of well-stained spindles, not only in the polar regions, but also in the equatorial region. These observations are consistent with the predicted result, lending more credence to the hypothetical mechanism of the stain proposed previously.

Agar gel was adopted into the standardised method, as it was the easiest to remove, and relatively easy to apply.

4) Concluding remarks

By investigating and standardising the procedure, adopting an Aluminium Sulphate wash, and by introducing a diffusion barrier, a protocol has been devised which is totally reliable in its production of well-stained extrafusal axons and extracapsular intrafusal axons from whole muscles. The staining of intracapsular axons is also greatly improved.

This method has been used to produce all the specimens in this study, and also in other subsequent studies of reinnervation. It has produced well-stained spindles from cat, rabbit and rat whole muscles (Plate 2.1).

Further developments were in progress, wherein muscles were embedded in the walls of an agar cylinder, through which contra-diffusing solutions of Ag and ammoniacal alcohol passed, and a pilot study, using this technique, produced very encouraging results.

CHAPTER THREE

CHAPTER THREE

THE CRUSH STUDY

EXPERIMENTAL DESIGN

Cat peroneal muscles were chosen for this study because this would allow correlation of the histological findings with the physiological results of Brown & Butler (1976). In addition, the normal innervation of these muscles has been extensively studied (Barker et al., 1979; Banks et al., 1982), and the peroneal nerve, being positioned superficially as it is at the knee, presents an ideal site for producing nerve lesions while causing the minimum of distress to the animal.

It was decided not to use the crushing technique adopted by Brown & Butler (1976). They crushed the nerve in three places, at 2mm intervals. This could mean that the reinnervating axons were regenerating through very different conditions. For example, if an axon was damaged at the most proximal crush, it would have to traverse three sites of disruption caused by crush and grow 4mm further than an axon damaged at the most distal crush site, which would have only one crush site to cross.

Instead it was decided to crush the peroneal nerve twice, in the same place, once from each side. This not only provides all the reinnervating axons with the same distance, and same conditions through which to grow, but also ensures that any irregularities in the forceps which might allow some axons to escape crushing, would not be applied to the same part of the nerve trunk.

The peroneal nerve was crushed in 12 adult cats (av. wt. 2.2 kg) with fine smooth-tipped watchmaker's forceps (Inox 4), for a total period of two minutes, one minute from each side (for operating conditions see Techniques). The animals were killed at intervals from 3 to 37 weeks later by an overdose of sodium pentobarbitone, and the peroneal muscles were removed and processed for the production of teased, silver preparations.

PRELIMINARY EXPERIMENTS

It is an essential prerequisite to know that the method of crush adopted produces total denervation of muscle spindles by myelinated axons.

Brown & Butler (1976) performed acute experiments on three animals 5 days after the crush, in which the peroneal nerve was stimulated proximally to the site of the lesion. Although no contractions were observed, it is unlikely that any contractions of intrafusal fibres in the centre of the muscle, would be seen. However, histologically, uniform Wallerian degeneration was observed.

In order to check the efficacy of the operating technique in the present study, an animal was killed one week after the crush operation; the nerve was processed for electron microscopy (see Techniques), and the peroneal muscles from both operated and contralateral sides were processed for the production of teased, silver preparations. On examination under E.M., there were no signs of any intact myelin sheaths, and axons showed signs of degeneration (Plate 3.1.B). On teasing the muscles from the operated side, no spindles containing myelinated axons could be found, although presumed autonomic axons accompanying capillaries were seen. The contralateral muscles, processed in the same receptacles, showed well-stained axons of a totally normal appearance.

On the basis of these two results, it was concluded that the method adopted for crushing the nerve produced total denervation of spindles, and subsequent analysis has not produced one spindle of normal appearance.

Rather than present the results in terms of days post operation, it is clearly more relevant to know, from the point of view of comparing results, how long the axons have entered and have been reinnervating the muscle. For the purposes of this study this is termed the Reinnervation Time (R.T.) (Barker & Boddy, 1980). In order to calculate the Reinnervation Time for any time period after the operation, and for any of the three peroneal muscles that were examined, it is necessary to

know the rate at which the fastest axons grow from the site of the lesion, and the time taken for the growing tips to start growing from the lesion site, i.e. the "latent period" described by Gutman, Guttman, Medawar and Young (1942).

Two experiments were conducted to calculate these values. The tibial nerve was crushed in two cats as described previously. One week later the peroneal nerve was crushed at the usual position in both animals. After another week, the animals were killed by an overdose of sodium pentobarbitone, and both peroneal and tibial nerves were removed and processed to produce silver-stained sections (see Techniques). On examination, the crush site could be seen (Plate 3.2.C). The proximal and distal sides of the site could be clearly differentiated; the proximal side showing the ordered arrangement of myelinated axons, and the distal side showing characteristic Wallerian degeneration (Plates 3.2.A & B).

To compensate for shrinkage, the lengths of nerve removed were measured in situ, and again after processing, with cotton thread.

On examination of all the sections taken from any one nerve, the most distal growing tip was identified (Plate 3.2.D), and the distance from this to the crush site measured. This process was repeated on the remaining three nerves. Another operator repeated the measurements to ensure validity (Dr M. Saito).

Knowing the time between operations, and the difference in distance between the crush site and the most distal growth cone for the tibial and peroneal nerves, it is possible to calculate the growth rate of the fastest growing axons. The average growth rate was found to be 3.2 mm/day, and the calculations from both animals were in close agreement.

As both the growth rate of axons, and the distance from the crush site to the most distal growth cone are known, the "latent period" can be calculated for the four nerves analysed. All four values were found to be in close agreement, with an average of 5.8 days.

The distances from the crush site to the points of nerve entry into the

three peroneal muscles in a 2.2 kg cat were found to be 15, 26 and 43 mm for Peroneus longus (PL), Peroneus digiti quinti (PDQ) and Peroneus brevis (PB) respectively. Hence the growing tips of axons are entering PL muscle 10 days after the crush and at 14 days and 19 days for PDQ and PB . The Reinnervation Time can then be calculated for each muscle by subtraction of these values from the post-operative time.

ANALYSIS

For the purposes of presenting the results, it will be assumed that axons which reinnervate spindles and produce terminals that are identifiable by virtue of their similarity in form and position to normal endings, are axons which, prior to the lesion, had produced the normal terminals in question. For example, a large-diameter axon that innervates the spindle in the equatorial region, producing regular spiralling around the intrafusal fibres, will be classified as a Ia axon forming a primary ending. Whether or not this is a valid assumption will be discussed later.

1) Identification of intrafusal fibre type

Although it is tempting to use clues provided by the innervation, (for example, the distribution of p_1 plates, or the difference in length of regular spiralling in the primary ending), to do so is to prejudge the process of reinnervation. Therefore only non-neural indications will be adopted.

The bag fibres and chain fibres are easy to differentiate by virtue of the relative diameters alone, and by the disposition of the equatorial nucleation.

There are two main criteria by which the identity of nuclear bag fibres may be determined. The first is the difference in distribution of elastic fibres around the poles; there are fewer and shorter elastic fibres around the bag₁ fibre than around the bag₂ (Gladden, 1976). However, this criterion can only be used when the elastic fibres have been differentially stained, and this does not always occur. The other criterion is that bag₁ fibres are frequently excluded from the intrafusal bundle in the equatorial region, and this dissociation is often detectable. Bag₁ fibres are also usually shorter and thinner than bag₂ fibres, but these differences were seldom usefully discernible.

2) Identification of nerve terminals

Adopting rigid criteria derived from normal spindles to identify reinnervating terminals is to some extent pre-empting the study. However it is anticipated that endoneurial tubes will remain intact in the crush study, so that axons will return to their original sites on the spindle, and it is assumed that reinnervating axons will be identifiable on the basis of their resuming a form of termination recognisable from its normal appearance.

What will follow is an outline of the features that were used to identify restored terminals and reinnervating axons

a) Identification of sensory terminals

i) Primary endings

These are normally supplied by the thickest axon innervating a spindle. Normally there is only one, but occasionally double primaries are found. The Ia axon branches characteristically, and forms regular spirals around the equatorial nucleation of the intrafusal fibres.

Criteria used for identification of Ia axons and primary terminals were as follows:

- 1) Largest axon supplying a spindle.
- 2) Characteristic branching on approaching the equatorial region.
- 3) Terminals distributed among the intrafusal bundle, in the region of the equatorial nucleation.

ii) Secondary endings

In the normal spindle, the secondary ending can be positioned in any one of three positions, on either side of the primary terminal. The supplying group II axon is normally the second largest axon to innervate the spindle, and it too undergoes branching, and usually supplies all three types of intrafusal fibre (Banks et al., 1982).

There are two forms of the normal ending; annulo-spiral and

flower spray.

Criteria for identification

- 1) In the case of S_1 secondaries, the second largest axon supplying the spindle.
- 2) Characteristic branching to supply all three types of intrafusal fibre.
- 3) Position of the terminals in the S_1 , S_2 or S_3 positions.

b) Identification of motor terminals

The gamma innervation

In the normal spindle this exclusively fusimotor innervation is represented by two forms.

i) Trail innervation

In the normal spindle, the trail ramifications are usually supplied by more than one axon. Barker, Stacey and Adal (1970) report that there were 2.6 myelinated axons per pole supplying trail endings, and 1.6 non-myelinated axons, when these are present. The terminals are located intracapsularly and juxtaequatorially. i.e. mainly on the S_2 region, but with extensions to the S_1 and S_3 regions. They may extend over the entire polar region and be the only ending present. The form of the trail ending is diffuse and multiterminal and can take the form of simple brush-like tapers, hooks, knobs etc., and they have characteristically long preterminal axons.

Criteria for identification

- 1) Diffuse multiterminal ramifications
- 2) Supplied by more than one axon.
- 3) Ramification centred about the S_2 position.
- 4) Long preterminal axons.

ii) The p_2 plate innervation

In the normal spindle these are characteristically supplied by a single thick axon. They have an elongated shape with an average

length of 72.9 μm . They have knob-like axon terminals, and though possessing a sole plate, there is no obvious Doyère eminence.

Criteria for identification

- 1) Length of plate at least 27 μm . This is the lower limit of the normal range.
- 2) Relatively thick supplying axon.
- 3) The characteristic form of the ending.
- 4) The absence of a Doyère eminence.

c) The skeletofusimotor (beta) innervation

i) The p_1 plate

This is normally supplied by collateral branches of axons supplying extrafusal endplates, and has a similar appearance, with the sub-neural apparatus producing a pronounced Doyère eminence.

The site of normal p_1 plates is usually very polar and the supplying axon frequently branches from an intramuscular nerve trunk, rather than entering with the main spindle nerve trunk.

Criteria for identification

- 1) The plates may sometimes be seen to be supplied by collateral branches of axons forming extrafusal endplates.
- 2) The form of the ending is likely to be similar to extrafusal endplates.
- 3) A prominent Doyère eminence is often visible.
- 4) The plate may be supplied by an axon entering the spindle other than through the main nerve trunk.

NERVE DIAMETER MEASUREMENTS

The diameters of axons were measured using a Zeiss micrometer eyepiece and a $\times 40$ objective. Where remyelination had occurred, the measurements were made at the three most proximal nodes, and the mean value was calculated. Where remyelination had not occurred, three readings, at 100 μm intervals, were taken from the most proximal part of the nerve.

The values obtained were converted into their equivalent diameters in fresh material, by multiplying by a factor of 1.5 (Stacey, 1969).

HYPERINNERVATION

1) Motor terminals

Due to the large numbers of small-diameter axons present in the poles of many reinnervated spindles, it is not often possible to trace axons for long distances with any confidence. An ending was therefore classified as hyperinnervated if it was supplied by more than one axon.

2) Sensory terminals

The larger diameter of the Ia and II axons frequently allows them to be traced back into the nerve trunks. This makes it possible to check if putative hyperinnervated sensory terminals are, in fact formed by branches of only one axon.

The distance which axons must remain separate before they are classified as hyperinnervating, is arbitrary, and was taken as 800 μm . Terminals which were supplied by more than one axon were not classified as hyperinnervated if these axons could not be traced 800 μm .

OBSERVATIONS AND RESULTS

Sensory Reinnervation

1) Restoration of primary endings

a) Overview of restoration

The earliest signs of reinnervation occur in muscle after 5 days R.T. (P.D.Q.), the axons having an average diameter of $3.6\ \mu\text{m}$. However the form of the innervation cannot be determined at this stage.

The remaining ten analysable capsules show no signs of reinnervation by Ia axons at this stage.

After 11 days R.T. (P.L.), 75% of spindles have received reinnervation by Ia axons of average diameter $3.0\ \mu\text{m}$, and the percentage return stays approximately at this level until 53 days R.T. The branching of the Ia axons can be seen, and the terminals are beginning to form.

There are two examples of the developing terminals being distributed mainly to the presumed b_2 fibres, with only a very small contribution to the b_1 and chain fibres. Pl.3.3.C shows such an ending.

Other spindles reveal that the reinnervation of chain fibres has begun, (Plate 3.3.A) but where this has occurred, both bag fibres appear to be in at least as advanced a state of primary restoration as the chain fibres. An example of bag₁ fibre reinnervation can be seen in Plate 3.3B. The presence of the ring may indicate that, already, abortive parts of the terminals are being resorbed.

Some of the spindles which lack innervation by Ia axons at this stage have other axons in the equatorial region. Of these some are presumed to be sympathetic axons. (Plate 3.4.A) Others are axons which enter and leave the spindle without making any apparent contact with the intrafusal bundle. (Plate 3.4.B)

There are also examples of presumed motor axons which twist around the intrafusal fibres in the equatorial region as they approach

their destinations in the poles; while others pass through without such circumvolutions (arrowed Plate 3.4.C).

At 18 days R.T. the most well-formed primary ending shows terminals on presumed bag₁, bag₂ and chain fibres (Plate 3.5.A). However other spindles at this stage show only rudimentary endings (Plate 3.5.B), and some still do not receive any innervation from Ia axons.

At 25 days R.T. abnormalities in the restoration of the primaries include the reinnervation of one of the bag fibres, whereas the other bag fibre has no innervation, or, if present, it is only rudimentary. Plate 3.6.A. illustrates such a spindle, where one of the two presumed bag₁ fibres present receives no terminals from the primary ending, despite having well-formed terminals on the bag₂ fibre and on the chains. There are two such spindles which show this abnormality. In another spindle, it is the bag₁ fibre innervation which is well-restored, while the bag₂ fibre is poorly developed. There is one example where chain fibres only are excluded from the primary ending, but there is another example where the chain fibres are the only intrafusal fibres to receive innervation from the Ia axon.

Plate 3.7 shows two typical examples of primary endings after 25 days R.T. In Plate 3.7.A. separate bag₁, bag₂ and chain systems are present, but no regularly-spaced spirals around the intrafusal fibres can be seen. Plate 3.7.B. shows a primary in which two bag systems are present, though neither of them is well-developed.

At 39 days R.T. the abnormalities mentioned before persist, and there is one example of both bag fibres being excluded from the ending. Other spindles now have a primary ending of more normal appearance (Plate 3.8.A).

By 53 days R.T. all spindles have received innervation by Ia axons, but even by 240 days R.T. (Plates 3.9.B & 3.10.) where the primary is well-developed, some abnormalities still occur.

Days P.O.	Days R.T.	Muscle	% Return () sample size	Mean axon diameter (μ m) () no. of measurements	Standard Deviation
19	5	PDQ	17 (6)	—	—
21	11	PL	75 (12)	3.0 (12)	0.2
28	18	PL	85 (20)	5.0 (27)	1.6
35	25	PL	70 (23)	5.4 (42)	1.1
49	39	PL	76 (17)	5.4 (18)	1.1
63	53	PL	100 (11)	5.4 (21)	0.9
84	74	PL	100 (14)	5.2 (24)	0.6
91	81	PL	100 (11)	5.1 (15)	0.7
112	102	PL	100 (19)	4.5 (33)	0.7
140	130	PL	100 (20)	4.9 (30)	1.0
196	186	PL	100 (18)	4.0 (15)	0.8
217	198	PB	100 (9)	4.5 (27)	1.0
259	240	PB	100 (18)	4.8 (15)	0.4

Table 3.1

SUPPLY OF Ia AXONS TO SPINDLES AFTER NERVE CRUSH.

b) The rate of return

The % return of Ia axons is shown in Table 3.1 and Fig 3.1. The earliest presence in a spindle capsule occurs after 5 days R.T.

(P.D.Q.) and after 11 days R.T., 75% of spindles have received Ia axons. The % of spindles reinnervated remains approximately at this level until 53 days R.T., at which time all spindles are supplied. Thereafter there is 100% reinnervation by Ia axons.

It is possible to infer from this data that up to 25% of Ia axons are suffering significantly greater damage during the crush procedure, than the remaining 75%.

c) The diameter of Ia axons

The change in diameter of Ia axons with R.T. is shown in Table 3.1 and Fig. 3.1. The axons which were measured supply single primary endings only. Myelination occurs between 39 and 53 days R.T.

It can be seen that at 11 days R.T., the mean axon diameter is 3.0 μm , which increases to 5.0 μm at 18 days R.T.

Thereafter the diameter remains at over 5.0 μm at 18 days R.T. There is no drop in diameter between 39 and 53 days R.T., indicating that any reforming, but indistinguishable, myelin sheath has not been included in the earlier diameter measurements.

After 102 days R.T., the Ia axons have an average diameter of less than 5.0 μm , and this persists even at 240 days R.T.

d) Hyperinnervation

Most reinnervated primaries are supplied by a single Ia axon, whose first branching is close to the equatorial region. However, examples of hyperinnervation are seen, at times ranging from 39 to 240 days R.T. There is a total of 7 examples of hyperinnervated primary endings. In four of these there is one axon which is of comparable diameter to the Ia axons supplying non-hyperinnervated spindles. This is accompanied by

up to three smaller diameter axons. (Plate 3.11.C) The remaining three examples are supplied by 2,3 and 4 axons all of a large diameter (Plate 3.10).

e) Distribution of reinnervated primary endings to intrafusal muscle fibres

As has already been noted, the distribution of terminals to bag₁, bag₂ and chain fibres shows some abnormalities.

Since it is recognised that different intrafusal muscle fibres mediate different parts of the spindle's responses, any discrepancies in the distribution of the primary ending may account, in part, for abnormalities in the physiological responses of reinnervated spindles.

To investigate this, bag₁, bag₂ and chain fibres were scored separately on a scale of zero to three; zero implying a complete absence of innervation, and three implying a well-developed ending, normal in appearance and extent.

On summing the scores within each time course, and then for all analysable spindles after 53 days R.T., it becomes apparent that there is no overall trend towards any one of the intrafusal muscle fibre types having an increased or decreased contribution from the primary. The only exception is during the early stages of reinnervation when bag fibres receive more innervation than chain fibres. However, individual spindles do show marked deficiencies in one or two of the three fibre types at all times R.T. investigated.

The differences in the spiralling of the primary terminal, around the two kinds of intrafusal bag fibre which occur in the normal spindle, can often be seen to be restored.

f) Abnormalities of reinnervation

A common abnormality in the form of the primary is the loose spacing of the spirals in the ending. Where this occurs, the pitch to diameter ratio is typically 1.4 (Plate 3.6.B), as compared to the

more usual form of ending which has a ratio of 0.28. (Plate 3.9.B)

The loose packing of spirals can also be seen in Plate 3.8.C.

Occasionally part of the primary ending terminates in a large swelling, (Plate 3.9.B) but the rest of the ending usually has a relatively normal appearance.

Where such abnormalities are present, presumed motor axons, which also terminate in spherical swellings, are usually found.

g) Position and extent of the primary ending

Most primary endings return to their normal position, centred about the widest capsule diameter, and forming terminals around the intrafusal muscle fibres in the regions of equatorial nucleation. (Plate 3.8.B)

One example, however, forms an ending which is centred to one side of the equatorial region, with the result that terminals are distributed mainly to the myotube region and more polar to this, while the region of equatorial nucleation receives very little innervation. This may however be caused by interaction with a presumed II axon which is positioned in a more equatorial situation than is normal.

The normal primary ending occupies a length of about 300 μm (Barker 1974). The extent of reinnervated primary endings is similar for well-restored endings. Though some occupy up to 400 μm for a single primary. Poorly restored primaries are usually of smaller extent.

h) Other axons in the equatorial region

During the early periods of reinnervation (17-24 days), presumed motor axons which enter the equatorial region and terminate in swellings are common.

There are examples of seven such axons entering the equatorial region, in the absence of a primary innervation, and forming spherical and club-shaped terminations. (Plates 3.12. A, B & C).

Others pass through the equator, having formed swellings, and then pass on through to the poles. (Plate 3.13.B)

Pls. 3.13. A, C&D show examples of other axonal swellings from presumed motor axons in the myotube and juxta-equatorial regions of spindles.

Days P.O.	Days R.T.	Muscle	% Return () sample size	Mean axon diameter (μm) () no. of measurements	Standard Deviation
19	5	PDQ	17 (6)	—	—
21	11	PL	18 (11)	2.7 (6)	0.2
28	18	PL	61 (18)	3.0 (39)	0.8
35	25	PL	69 (13)	3.2 (54)	0.8
49	39	PL	76 (17)	3.5 (27)	0.9
63	53	PL	82 (11)	3.5 (27)	0.7
84	74	PL	83 (12)	2.8 (24)	0.6
91	81	PL	71 (7)	3.5 (9)	0.2
112	102	PL	64 (14)	3.8 (18)	1.0
140	130	PL	77 (9)	3.5 (9)	0.8
196	186	PL	75 (16)	2.5 (12)	0.4
217	198	PB	71 (7)	2.3 (15)	0.6
259	240	PB	70 (10)	2.7 (6)	0.5

Table 3.2

SUPPLY OF II AXONS TO SPINDLES AFTER NERVE CRUSH.

2) Restoration of secondary endings

a) Rate of return

The results for the restoration of secondary endings are shown in Table 3.2 and Fig. 3.2.

It can be seen that, although the earliest II axons to return do so at 5 days R.T., the same time as the first of the Ia axons, it takes till 20 days R.T. before the percentage of spindles receiving reinnervation by II axons approaches its final level of approximately 70%, which is lower than the normal frequency of greater than 85% (Barker 1974).

b) Diameter of II axons

The diameter of the earliest measurable axons is 2.7 μm , comparable to the diameter of Ia axons at this stage. Thereafter the diameter increases to 3.8 μm at 100 days R.T., but then diminishes till, at 240 days R.T., the average diameter is the same as it is at 11 days R.T. i.e. 2.7 μm .

c) Hyperinnervation

One instance of hyperinnervation involving one thin supernumerary axon has been seen.

d) The form of the ending

In the normal animal, secondary endings are described as being more dispersed than the primary and as appearing as fine tendrils forming a delicate tracery (Barker 1974).

The most usual form of the ending is annulospiral, but a less regular flower-spray ending occurs in approximately a third of cases.

Pls. 3.14 & 3.15 show restored secondary endings, and it can be seen that they regain the fine tendril-like appearance of the normal

ending, but that they are very diffuse and lacking in elaboration. Pl. 3.15.A. shows part of a well-stained, well-restored primary ending, in addition to the secondary, indicating that the paucity in the elaboration of the ending is actual rather than the result of a deficiency in the staining.

Due to the very sparse spray-like nature of restored endings, it has, in most cases, not been possible to determine, with any degree of certainty, the distribution of the terminals to the intrafusal fibres.

e) Position and extent of endings

As in normal spindles, most restored secondary endings occur in the S_1 position defined by Boyd (1962), but are much less extensive; the largest occupy a length of about 200 μm , which is outside the range of normal endings. (250 - 700 μm) (Plate 3.15.B)

f) Interactions with other axons

Secondary endings are usually restored in their normal position, either side of the equatorial nucleation, even in the absence of reinnervation by Ia axons.

There is an example of a presumed II axon forming a terminal which encroaches on the equatorial nucleation causing branches from a presumed Ia axon to be rejected.

There is however another example where the primary ending has been formed to one side of the equatorial nucleation forcing a presumed II axon to abort in the S_1 region.

Two examples have been noted where a branch from an S_1 secondary, lying to one side of the primary region, travels through the equator and forms terminals in the other S_1 position. In one case, the primary terminals are present, and, in the other, the Ia axons have not arrived.

Days P.O.	Days R.T.	Muscle	No. poles examined	Mean number of axons per pole.	
				\bar{x}	S.D.
21	11	PL	19	4.5	2.3
28	18	PL	36	6.9	3.4
35	25	PL	17	8.9	2.8
49	39	PL	24	8.6	2.8
63	53	PL	4	11.8	4.8
84	74	PL	24	8.1	3.0
112	102	PL	10	11.9	2.7
140	130	PL	21	8.0	2.6
196	186	PL	24	8.5	2.7

Table 3.3

SUPPLY OF MOTOR AXONS TO POLES OF PERONEUS LONGUS SPINDLES AFTER NERVE CRUSH.

Motor Reinnervation

One of the effects of a nerve-crush injury is that the damaged axons branch and send out several sprouts. To quantify the effect this has on the motor input of muscle spindles, the number of axons which terminate on any one spindle pole was counted.

In many spindles the reinnervating axons have produced such chaotic and complicated terminations that no realistic estimate can be made. The true extent of hyperinnervation may therefore be greater than that indicated by these measurements. The results are shown in Table 3.3.

In normal spindles from P.L., the mean number of motor axons is 4.08 (Barker, Stacey & Adal 1970), and this level is reached within 11 days R.T. Thereafter the amount of motor innervation received reaches a level of between two and three times normal, a level which does not diminish even after 240 days R.T.

Restoration of motor endings

The intrafusal p_1 plates are supplied by collateral branches of Beta axons (Barker, Stacey & Adal 1970). It is therefore of interest in assessing the restoration of p_1 plates to know the state of reinnervation of extrafusal endplates at the same interval R.T.

1) Restoration of extrafusal endplates

The first signs of extrafusal endplate reinnervation occur at 2 days R.T., but the first photogenic examples are seen at 11 days R.T. (Pl. 3.16A-C), where the axon sprout has arrived at the sole plate and has started to branch. Pl. 3.16.H. shows the characteristic sole plate with its nuclei in profile, receiving an axon after 11 days R.T.

With time, the extrafusal endplate reassumes its characteristic formation (Plate 3.16 D,E & G).

No examples of hyperinnervated extrafusal endplates have been found. The only abnormality is shown in Pl. 3.16.F., where the ending has clubbed

Days P.O.	Days R.T.	Muscle	Mean diameter of axons supplying P1 plates (μm)			Mean diameter of axons supplying P2 plates (μm)			Mean diameter of axons supplying Trail endings. (μm)		
			\bar{x}	n	S.D.	\bar{x}	n	S.D.	\bar{x}	n	S.D.
28	18	PL	1.2	5	0.5	1.2	4	0.1	0.9	2	-
35	25	PL	1.0	10	0.3	1.1	1	-	2.4	8	0.5
49	39	PL	1.5	11	0.8	1.5	1	-	1.4	6	0.6
63	53	PL	2.1	8	0.4	2.1	6	0.5	2.7	4	0.6
84	74	PL	1.8	12	0.5	1.8	7	0.5	2.0	8	0.4
112	102	PL	1.6	13	0.9	1.7	11	0.5	1.8	9	0.6
140	130	PL	1.4	12	0.3	1.7	6	0.4	2.0	8	0.3
196	186	PL	1.0	10	0.2	1.5	5	0.4	1.5	6	0.4
217	198	PB	0.9	6	0.1	1.3	7	0.2	1.2	4	0.3
259	240	PB	1.2	5	0.4	2.2	2		1.0	3	0.05

SUPPLY OF MOTOR AXONS TO SPINDLES AFTER NERVE CRUSH

Table 3.4

and hooked terminations, possibly indicating a degenerating endplate.

2) Restoration of p_1 plates

a) Overview of restoration

As extrafusal endplates start to be reinnervated at 2 days R.T., it is probable that p_1 plates will also be formed at this stage. However the first signs of reinnervation of p_1 plates occur at 5 days R.T. Pl. 3.17.A. shows a pair of p_1 plates after 11 days R.T., in which the axon has started to branch on the sole plate. Pl. 3.17.B. shows an endplate in profile, at a similar stage of restoration.

Most p_1 plates continue to mature in a similar way to extrafusal endplates until at 102 days R.T., they have a normal appearance.

b) Diameter of beta axons

The change in diameter of beta axons supplying p_1 plates can be seen in Table 3.4. As is the case with Group II sensory axons, the diameter increases over the first 100 days R.T., only to decrease to a value similar to its first measurable size at 240 days R.T.

c) The usual pattern of restoration of p_1 endplates

The normal beta innervation of intrafusal fibres terminates, in the majority of cases, in a single endplate. This is also seen in reinnervated material. Pl. 3.18.B. shows an example of a well-restored p_1 plate which has a very similar appearance to the reinnervated extrafusal endplate shown below it. (Plate 3.18.C.)

The collateral nature of p_1 innervation is confirmed by the example shown in Pl. 3.18.A., where the extrafusal endplate (e), is supplied by a branch of the axon (ax) which goes on to form a classical p_1 plate on a spindle (sp).

Some axons terminating in single endplates do not form the elaborate ending shown in Pl. 3.18.B (see Pl. 3.17 C & E). Where it is possible

to discern which intrafusal fibre has received the p_1 innervation, it is usually the bag₁ fibre, as in the normal spindle.

Beta axons in normal spindles sometimes branch and produce two adjacent p_1 plates on the same intrafusal muscle fibre. This form of termination is usually restored to its normal appearance, (Pl. 3.19.A, B & C), though there are three examples of these paired p_1 plates being distributed to different intrafusal fibres (Pl. 3.17.F.).

One example has been found where the axon supplying a typical Y-shaped double p_1 plate configuration, is accompanied by a much finer axon which terminates in a large irregular mass which is not on the site of the p_1 plates (Plate 3.20.F.).

Groups of more than two p_1 plates do occur in reinnervated material. Pl. 3.21.A. shows a complex of three p_1 plates. The upper two are supplied by a single thick axon, but they are also visited by an axon which returns up the supplying nerve trunk without making any contribution to the ending. The lower plate is supplied by two fine axons.

Pls. 3.21.B & C show examples of other multiple p_1 terminals; Pl. 3.21.B. shows an example formed from a single axon, Pl. 3.21.C. an example supplied by four axons.

d) Hyperinnervation

Hyperinnervation is a common abnormality. The most usual form occurs when two axons of a similar diameter contribute terminals to the same endplates. Pls. 3.22.A,B & C show examples of this form, ranging from 39 to 249 days R.T., indicating that resorption of supernumerary axons, if it occurs at all, may be a very slow process.

Sometimes one of the pair of axons is considerably smaller than the other. Occasionally a second axon arrives at a sole plate and terminates in spherical swellings (Plate 3.20 D&E), suggesting its non-acceptance by the sole plate.

One example has been seen where the sole plate is reinnervated by

three separate axons (Plate 3.17.E), all of which terminate in spherical swellings.

3) Restoration of p_2 plates

a) Overview of restoration

The earliest that p_2 plates are seen is at 11 days R.T., i.e. six days later than the p_1 plates. This may be due to difficulty in distinguishing p_2 plates in the early stages of their restoration. At 11 days R.T. the ending has a simple form, consisting mainly of tapers, and this frequently matures into a form easily recognised as a normal p_2 plate.

As with p_1 plates, the endings are distributed mainly to the bag_1 fibres.

b) The diameter of gamma axons supplying p_2 plates

The change in diameter of gamma axons supplying p_2 plates can be seen in Table 3.4. At all intervals R.T. the average diameter is at least that of the beta axons supplying p_1 plates, and after 74 days, it is always larger.

c) The length of p_2 plates

The average length of p_2 plates which have resumed a normal appearance, is 67 μ m, but while the terminals are still elaborating, the average length is considerably smaller, typically 40 μ m.

d) Hyperinnervation

Again hyperinnervation is a common abnormality. Usually this consists of a thick axon being shadowed by thinner axons, up to four in number, which contribute to the ending (Pl. 3.23. D & E).

e) Other abnormalities

Sometimes branching of the supplying axon occurs. Pl. 3.23 .F. shows

the simplest form of this, where one branch is found near the endplate; a form which does not occur in normal spindles. There are however examples in which up to five branches are formed and contribute to the plate. This does not happen normally. In some cases, thin accompanying axons terminate in an axonal ball within the p_2 site itself.

Occasionally several axons of apparently independent origin, supply a presumed p_2 site (Plate 3.24.A.). The terminals formed in this case are similar to the p_1 endplates, suggesting possible innervation by Beta axons. There is one example where some of the very fine axons, contributing to a p_2 site, have been found to originate from an axon supplying a p_1 plate.

There is another example of a presumed p_2 site which is visited by fine axons (Plate 3.24.B). One of these terminates in a hook. The others end in very fine tapers, apart from one thick axon which contributes a fine branch to the p_2 site and then leaves.

Ultraterminal fibres which run on from the end of a plate and terminate in a ball of axoplasm are sometimes seen in normal material, and are certainly present in restored plates (Plates 3.25A & B and 3.24.C).

4) Trail terminals

Positive identification of trail endings is difficult; in the early stages, as their form can easily be confused with those of other terminals, and in later stages because the juxtaequatorial region which they innervate is frequently obscured by large numbers of axons.

The earliest presumed gamma axons forming trail terminals, are seen at 5 days R.T. At 11 days R.T. more elaborate trail ramifications have been formed (Plate 3.26.A, B & C). Later stages of reinnervation are shown in Plate 3.27.A, B, C & D.

Recurrent axons which emerge from the site of a trail ending, and leave the spindle, are occasionally found in normal material, but are fairly common in reinnervated spindles.

Days P.O.	Days R.T.	Muscle	% of spindles having an invading axon. () sample size	Mean diameter of of invading axons(μ m)		
				\bar{x}	n	S.D.
19	5	PDQ	0 (5)	-	-	-
21	11	PL	0 (12)	-	-	-
28	18	PL	22 (18)	1.9	4	0.9
35	25	PL	29 (21)	2.4	5	0.4
49	39	PL	44 (16)	2.5	2	0.3
63	53	PL	31 (13)	3.0	4	0.6
84	74	PL	53 (15)	2.6	5	0.7
112	102	PL	55 (11)	1.8	7	0.7
140	130	PL	43 (14)	1.7	5	0.2
196	186	PL	36 (11)	1.2	2	0.2
217	198	PB	50 (12)	1.0	3	0.1
259	240	PB	41 (17)	-	-	-

Table 3.5

INNERVATION OF SPINDLES BY PRESUMED MOTOR INVADING AXONS AFTER NERVE CRUSH

Trail ramifications are seen to reinnervate mainly bag₂ and chain fibres, but sometimes bag₁ fibres receive a contribution from the ending.

5) Visiting axons

Examples of axons which enter the spindle and then leave it , having made terminations, have been noted from 18 to 240 days R.T.

Some of the formations these axons produce are shown in Pls.3.28,.29&.30. The occurrence of these axons has been calculated and is shown in Table 3.5. They reach their greatest incidence at 102 days R.T., and there is no marked decrease after that.

The only form of innervation that produces an axon which leaves the spindle, having made a termination, is the trail ending. Any axons therefore which have formed trail-like endings before leaving the spindle, have not been included in the quantification.

The nature of these axons will be discussed later.

Days P.O.	Days R.T.	Muscle	Percentage return of Ib axons () sample size.	Mean diameter of Ib axons (μ m)		
				\bar{X}	n	S.D.
21	2	PB	0(1)	-	-	-
19	5	PDQ	50(4)	3.3	2	0.05
28	18	PL	67(6)	5.1	4	0.8
35	25	PL	89(9)	6.0	3	0.5
63	53	PL	80(5)	2.9	2	0.1
84	74	PL	100(1)	-	-	-
112	102	PL	80(10)	4.0	4	0.8
140	130	PL	100(8)	2.9	2	0.4
196	186	PL	100(2)	3.2	2	0.1

Table 3.6

SUPPLY OF Ib AXONS TO GOLGI TENDON ORGANS AFTER NERVE CRUSH.

Restoration of Golgi tendon organs

The presence of Ib axons innervating tendon organs is first noted after 5 days R.T., but, if the small sample at 74 days R.T. is ignored, 100% restoration is only achieved at 130 days R.T. (Table 3.6), taking longer therefore than the Ia axons.

The terminals are restored to their normal appearance. There is only one example of hyperinnervation when five afferents return to produce a normal-looking ending (Plate 3.31).

Paciniform corpuscles

A total of four paciniform corpuscles has been found. Three of these are innervated by a single axon (average diameter $1.8\mu\text{m}$) (Plate 3.32). One however is situated adjacent to a Golgi tendon organ, and is innervated by a branch of a single axon, the other branch of which innervates the tendon organ and forms there a normal spray of terminals.

SUMMARY AND COMMENT

Extrafusal alpha motoneurons return to the muscle and form motor endplates very quickly following nerve-crush, being seen after 2 days R.T. In the muscle nerve these alpha motoneurons have a range of diameters comparable to that of the Ia axons, but it is only after 11 days R.T. that the return of Ia axons reaches a comparable level of reinnervation. The skeletofusimotor p_1 plates are established after 5 days R.T., when only 17% of spindles receive Ia axons, and trail-like ramifications are present in the spindle poles at the same early stages of reinnervation.

These observations indicate that following nerve-crush, the sensory innervation is slower in returning than the motor. Within the sensory component, Ia axons return at a faster rate than II axons, as after 11 days R.T., the return of Ia axons has reached an interim level of effectively 100%, whereas that of the II axons has only reached one third of its final level. Within the motor component, the extrafusal motor fibres return first, followed by skeletofusimotor axons and the gamma component. In the case of both motor and sensory axons, the larger diameter axons return more quickly than the smaller.

It has been noted that the level of reinnervation by Ia axons reaches a stable level of approximately 70% after 17 days R.T. This level is maintained until 53 days R.T., at which time all spindles receive innervation by Ia axons, a condition which persists at all subsequent time intervals R.T. It would seem likely that the explanation for this occurrence is that a proportion of axons and their supporting tissues within the peroneal nerve, are sustaining significantly greater damage than the rest, and that this is delaying their return. Certainly the form or extent of the damage is not continuously variable, as the pattern of return is marked by a discrete "second wave" rather than by a gradual increase in the return. An example of such non-continuous damage would be a severance of the axon and its endoneurial tube, which would be more likely to happen in large diameter axons. If this damage is a function of the diameter of the Ia axons, then other

large-diameter axons within the peroneal nerve would also be affected.

In the case of the Ib axons the modal value for the distribution of diameters is smaller than that for Ia axons, and therefore a smaller " second wave " would be expected. It can be seen from Table 3.6 that, although the sample numbers are small, there is a plateau of reinnervation between 18 and 53 days R.T. before 100% reinnervation is restored.

Another population of large-diameter axons in the peroneal nerve are the alpha motoneurons. It would not be possible to see a " second wave " of reinnervation in the extrafusal fibres in this study, but it is possible that fractured endoneurial tubes could provide an explanation for the observations of axons visiting spindles, making attempts at endings, and subsequently leaving. The explanation would be as follows: the endoneurial tubes of alpha motoneurons are ruptured and the axons themselves are effectively " cut ". The regenerating axons branch and some branches grow down endoneurial tubes which lead them to the spindle, where they attempt to form an endplate, and then go on to leave the spindle, and presumably eventually form an extrafusal endplate. Such axons have been termed " alpha invaders " (Barker & Boddy, 1980).

If these alpha invaders make functional connections with the intra-fusal fibres, then it might be expected that the amount of beta innervation would increase following nerve-crush.

As there are usually 6 or 7 intrafusal fibres which mediate the static response (bag_2 and chains), as compared to the usual one which mediates the dynamic response (bag_1), then it might be expected that the " invading " axons would have a predominantly static effect. Brown & Butler (1976) have reported an increase in the beta static innervation after nerve injury. After nerve section there are potentially more opportunities for these invading axons to reach spindles than is the case after crush, and Brown & Butler (1976) also report that the increased static beta innervation is more marked after cut than after crush.

The physiological investigations of Ip, Vrbova & Westbury (1977) found that the dynamic component of the responses of reinnervated spindle primaries was the first to be restored. Two explanations of this result are either that the transduction mechanism of the primary ending matures in such a way that the dynamic component of the response returns first; or that the primary ending is first restored on that part of the spindle which mediates the dynamic response, i.e. the bag₁ fibre. The lack of any consistent observation that the bag₁ fibre receives elaborated terminals from the primary ending before the other intrafusal fibres, indicates that the former explanation is the more likely.

Support for this interpretation of the results of the present study has been given by the studies of Scott (1982) and Hyde & Scott (1983), who propose that the abnormal responses of reinnervated spindles are caused by an increase in the pace-maker threshold which lowers as the ending matures.

Hyperinnervation is present in all forms of reinnervated intrafusal ending. However, its presence is noted even after the longest periods R.T. This phenomenon may be due to the persistence of the branches formed at the site of the injury, or to the usual branching of a supplying axon occurring more proximally than normal. Another explanation is that branches from one endoneurial tube are growing down another tube, with the original axon still there. This may also contribute to the increased beta innervation described by Brown & Butler (1976).

The mechanism which eliminates polyneuronal and multiple neuronal innervation in extrafusal fibres does not appear to operate intrafusally.

The diameters of axons reinnervating spindles remain lower than normal at all periods R.T. A relative difference in the diameters of Ia and II axons persists, but the overall reduced diameter makes the identif-

ication of other axons by means of their diameter impossible. This is consistent with the observations made by Brown & Butler (1976), Ip et al. (1977) and Hyde & Scott (1983).

Brown & Butler (1976) concluded that spindle reinnervation was a highly specific process. The results of this study indicate that, in the majority of cases, both the primary and secondary endings are restored on their original positions on the spindle, and that the motor innervation resumes its usual distribution. The observation that a Golgi tendon organ and a pacini-form corpuscle share a common axon, however, may indicate that there may not be a rigid specificity of axon function.

The fact that there are errors in the positioning of primary and secondary terminals may indicate that site specificity possibly diminishes with time, in the absence of the correct innervation.

CHAPTER FOUR

CHAPTER FOUR

SUPPLEMENTARY STUDIES

SECTION ONE: THE REINNERVATION OF MUSCLE SPINDLES AFTER NERVE SECTION

Introduction

Following the crush study, in which regenerating axons faced little difficulty in reinnervating muscle spindles, it was decided to conduct a series of experiments in which the reinnervation could be studied after nerve section. This would provide a more severe test for the specificity of reinnervation. It was also anticipated that the latent period following this operation would be longer, so that the length of time a spindle was left without its correct innervation would be greater, and any time-sensitive elements within the conditions which cause site-specificity might become apparent.

It was hypothesised from the previous study that the presence of the presumed alpha invaders might have been caused by the severance of endoneurial tubes. In a situation where all the endoneurial tubes are severed, it might be expected that the occurrence of such axons would increase, and if the teasing strategy were altered to include more of the surrounding extrafusal muscle fibres, it might be possible to identify a presumed alpha invader as an axon which does eventually produce an extrafusal endplate.

Methods

So that correlation could be made with the crush study it was decided to use peroneus longus muscle, and to cut the peroneal nerve at the knee, 15 mm. from the nerve entry to P.L.

As one of the purposes of this study is to provide histological correlation with the studies of Brown and Butler (1976), and in the absence of professional surgical expertise in nerve suturing, no attempt was made to appose the cut ends of the nerve. This resulted in the stumps lying with a gap of approximately 1mm. between them.

The right common peroneal nerve was cut at the knee using the usual operating conditions (see Techniques), in six adult cats of average weight

2.3 kg. The animals were killed at 5,6,8,10,12 and 30 weeks after the operation with an overdose of sodium pentobarbitone. The peroneus longus muscles were removed from both sides and processed for the production of teased, silver-stained preparations (see Techniques). The muscles from the left-hand side were used to check the efficacy of the staining.

Table 4.1

Axons present in spindles	-	+	+	-	+	+
Sample size	15	12	13	8	9	15
Axons present in GTOs	-	+	+	-	+	+
No. of GTOs receiving axons	0	4	8	0	5	8
Sample size	6	9	8	5	6	8
Extrafusal motor endplates restored	-	+	+	-	+	+
Axons present in nerve trunks	-	+	+	+	+	+
Weeks P.O.	5	6	8	10	12	30

Innervation after nerve section.

innervation by these large axons.

One of these large axons (diameter $3.5\ \mu\text{m}$) branches within the capsule and terminates in the equatorial region of the spindle, in the form of thin tapers disposed longitudinally upon the intrafusal bundle. The other large-diameter axon ($4.0\ \mu\text{m}$) enters a spindle, turns around, returns to its point of entry, then leaves without having branched or made any recognisable terminations.

At eight weeks P.O., out of a sample of thirteen spindles, five lack such large-diameter axons, while another five receive them, and 3 of these branch within the spindle, producing longitudinal tapering endings around the equatorial region; two of these produce branches which leave the spindle. Two spindles receive thick presumed Ia axons, which enter the spindle, then leave without making any terminals (Plate 4.3 A). Fig. 4.1 shows one example where the axon (diameter $4.7\ \mu\text{m}$) travels for nearly one millimetre along the intrafusal bundle, only to turn around and leave without having branched or made any terminals.

At 10 weeks P.O., no axons are present within spindles, but at 12 weeks P.O. six spindles out of a sample of nine are supplied by these large-diameter axons. They all have the same basic " longitudinally-oriented " form of the ending, as described above, but three at this stage form a few regular half-rings, similar to the normal primary as well (Plate 4.1 A & B). Two which do not form such spirals both produce at least one branch which leaves the spindle. Small bulbous terminations are sometimes seen in the equatorial region (Plate 4.3 E).

The remaining spindle is innervated in the equatorial region by two large axons (diameters $3.1\ \mu\text{m}$ and $3.2\ \mu\text{m}$); these approach the equatorial region from opposite directions and there they adopt the longitudinally-oriented form of termination, overlapping one another, but no signs of regular spirals can be seen.

At 30 weeks P.O., there are two examples in a sample of fifteen spindles, where these large-diameter axons produce the regular spirals of the normal primary ending, but these are still of very small extent and they occur in the

OBSERVATIONS AND RESULTS

As can be seen from Table 4.1 , 10 weeks after the cut operation, no nerves have returned to any spindles or Golgi tendon organs within the muscle, even though their presence has been observed after 6 weeks and 8 weeks P.O. (post operation).

The contra-lateral P.L. muscle from the 10 week cut animal, reveals well-stained spindles, indicating that the observed absence of axons within spindles and tendon organs is an actual occurrence rather than a deficiency in staining.

This implies that the cut operation does not provide a uniform environment for regenerating axons, presumably because of the variability in the success rate of the two cut faces of the nerve forming a union. This means that the reinnervation process cannot be expressed in terms of Reinnervation Time as in the crush study, and that results from any one animal are not strictly comparable to any other, as the period during which the muscle was denervated is not known. Nevertheless it is still possible to describe the innervation of spindles after cut injury, without being able to relate the exact sequence of events.

Sensory restoration

A great difficulty in analysing the restoration of sensory endings after the cut lesion, is that the equatorial region is frequently obscured by a tangle of very fine axons (Plate 4.1 A,B,C & D). When traceable, these axons are often seen to turn around and cross the equatorial region several times before, either leaving the spindle, or terminating in fine tapers. On many occasions this is the only form of innervation that is present in the equatorial and juxta-equatorial regions.

Most of these axons are of very small diameter (usually less than $1\text{ }\mu\text{m}$); presumed sensory axons are identified in spindles after cut lesions, by their comparatively large diameter (Plate 4.2 D). In 6 weeks P.O. material, two spindles out of a sample of twelve can be identified as receiving

innervation by these large axons.

One of these large axons (diameter $3.5\ \mu\text{m}$) branches within the capsule and terminates in the equatorial region of the spindle, in the form of thin tapers disposed longitudinally upon the intrafusal bundle. The other large-diameter axon ($4.0\ \mu\text{m}$) enters a spindle, turns around, returns to its point of entry, then leaves without having branched or made any recognisable terminations.

At eight weeks P.O., out of a sample of thirteen spindles, five lack such large-diameter axons, while another five receive them, and 3 of these branch within the spindle, producing longitudinal tapering endings around the equatorial region; two of these produce branches which leave the spindle. Two spindles receive thick presumed Ia axons, which enter the spindle, then leave without making any terminals (Plate 4.3 A). Fig. 4.1 shows one example where the axon (diameter $4.7\ \mu\text{m}$) travels for nearly one millimetre along the intrafusal bundle, only to turn around and leave without having branched or made any terminals.

At 10 weeks P.O., no axons are present within spindles, but at 12 weeks P.O. six spindles out of a sample of nine are supplied by these large-diameter axons. They all have the same basic " longitudinally-oriented " form of the ending, as described above, but three at this stage form a few regular half-rings, similar to the normal primary as well (Plate 4.1 A & B). Two which do not form such spirals both produce at least one branch which leaves the spindle. Small bulbous terminations are sometimes seen in the equatorial region (Plate 4.3 E).

The remaining spindle is innervated in the equatorial region by two large axons (diameters $3.1\ \mu\text{m}$ and $3.2\ \mu\text{m}$); these approach the equatorial region from opposite directions and there they adopt the longitudinally-oriented form of termination, overlapping one another, but no signs of regular spirals can be seen.

At 30 weeks P.O., there are two examples in a sample of fifteen spindles, where these large-diameter axons produce the regular spirals of the normal primary ending, but these are still of very small extent and they occur in the

myotube region (Plate 4.3 D). There are four examples of a single large-diameter axon branching and producing longitudinal tapering terminals, and a further two examples of this formation that have a branch which leaves the spindle (Fig. 4.2).

One spindle receives a large-diameter axon which enters and leaves the spindle having travelled approximately 600 μm over the intrafusal bundle towards the equator, without branching or forming terminals (Fig. 4.3).

Another example of a large-diameter axon is illustrated in Fig, 4.4 , where it enters a spindle in the equatorial region and branches (a). The finer of the two branches travels approximately 100 μm along the intrafusal bundle, then divides again (b), one fork returning to the point of entry and then going out of the spindle, the other travelling a further 100 μm before it too turns and leaves the spindle. This occurs above the equatorial nucleation. The thicker of the two original branches travels approximately 100 μm before it branches again (c). This branch leaves the spindle, then travels in a polar direction for a further 300 μm before returning to the point where it split from the original axon, whereupon it produces a small branch and leaves the spindle again. The second of the original branches, after leaving the spindle, divides twice more, and one of the resultant branches returns to the spindle at (d).

In the examples where regular spirals are produced by large axons, these are all distributed to a bag fibre, as judged by the diameter of the spirals, but apart from that, the coursing axons and axon branches appear to be distributed indiscriminately over the intrafusal bundle.

In those spindles examined where axons have been present, there has always been a large number of very fine axons in the equator. It is possible that some of these are branches of sensory axons and it is therefore impossible to estimate the relative degree of innervation by such large-diameter axons, nor is it possible to estimate hyperinnervation, if it is effected by very fine branches. Hyperinnervation would only be detected in a situation where all the hyperinnervating axons are appreciably larger than the very fine ones in the

equatorial region. Only one example of hyperinnervation has in fact been found, where the spindle is supplied by two large axons.

One example has been seen in which terminals resembling those of secondary endings are found in the S_1 position. These are formed from an axon 1.8 μ m in diameter.

Motor restoration

1) Overview of restoration

In contrast to the highly neurotised picture of the equatorial region, the poles of the muscle spindles are relatively poorly supplied by axons. At 6,8,12 and 30 weeks P.O. features of reinnervation are seen.

At these stages, axons are seen terminating in swellings, frequently paired (Plate 4.3 C) as well as in tapers. These axons often show varicosities (Plate 4.2 C). Axons which enter the spindle and leave it without having branched, as well as those which have branched before leaving, are present in all spindles.

2) Restoration of extrafusar endplates and p₁ plates

In 6 weeks P.O. material no extrafusar endplates can be recognised. The only axons which can be seen extrafusally are very fine, with varicosities. Intrafusally, presumed p₁ sole plates are found, and where these receive innervation, it is usually by a single fine axon which terminates in a swelling. These supplying axons are also frequently varicose. One example can be seen where one thick axon accompanied by several thin ones supplies a single sole plate, but they all end in swellings.

At 8 weeks P.O. extrafusar endplates are recognisable, taking the form of very fine axons which divide to produce two or three tapers within the plate (see Plate 4.4 E). One example occurs of an extrafusar endplate receiving innervation by two axons, both of which contribute to the ending. Elongated club-shaped terminations can also be seen extrafusally (Plate 4.4 C). Another of the extrafusar endplates produces rings and swellings as well as an ultraterminal sprout (Plate 4.4 A). Most p₁ plates still have a rudimentary form, usually ending in a swelling or in a simple branch within the sole plate. No examples of hyperinnervation are found.

At the remaining stages of 12 and 30 weeks P.O., the extrafusar endplates usually have more extensive ramifications within the sole plate,

but there are examples where the endplate still has a rudimentary form (see Plate 4.4. F). The extrafusal endplates are almost exclusively supplied by single axons. At 12 weeks P.O., examples of large terminal swellings are seen situated on extrafusal muscle fibres (Plate 4.3 B). Some examples of ultraterminal sprouting are present even at 30 weeks P.O. (Plate 4.4 B).

Usually p_1 plates are also restored with single axons, but the branching within the endplate is poor, and the axon sometimes terminates in a swelling. Where identification is possible, the p_1 plates are restored on bag_1 fibres.

Three examples of ultraterminal sprouting of a p_1 plate onto an adjacent extrafusal fibre can be seen (Plate 4.4 D).

3) Restoration of p_2 plates and trail endings

One elongated (46 μm) plate-like ending occurs in 6 weeks P.O. material, supplied by a single axon, but apart from this, the only other two examples of presumed p_2 plates are found at 30 weeks P.O. (average length 43 μm). One of these is formed by an axon which has previously visited an adjacent spindle without branching. Identification of the intrafusal fibre type, on which the p_2 plate is formed, is not possible in any of these examples.

The chaotic formations of axons in the juxta-equatorial region make confident identification of trail endings in the cut study very difficult, but trail-like configurations are present at 6,8,12 and 30 weeks P.O. Sometimes branches from these are seen to leave the spindle. Where identification is possible, trail endings are distributed mainly to chain fibres.

4) Invading axons

In all spindles where axons can be traced in their entirety, there are examples of axons entering the spindle and leaving without making terminations. Some pass through the equatorial region, and others are confined entirely to the poles.

Other axons entering the spindle, branch and produce terminations before

one or more of the branches leave the spindle; some retrace their path up the supplying nerve trunk and others leave via a different one (Figs 4.5, 4.6, and 4.7).

The form of one of these invading axons is shown in the example (Fig. 4.8) where a single axon enters a spindle having divided, and one branch forms tapering and bulbous terminations on the intrafusal bundle. The other branches leave the spindle as a bundle, and one of them goes to an adjacent extrafusal muscle fibre and forms an endplate. This axon is certainly of motor origin and is probably an alpha invader, the existence of which has been postulated as a result of the crush study.

5) Restoration of Golgi tendon organs

Axons are present in four tendon organs out of nine, at 6 weeks P.O., and in all but one case in the 8, 10 and 30 weeks P.O. material.

At both 6 and 8 weeks P.O. the axons terminate in tapers, and it is only at 12 weeks P.O. that a normal appearance is resumed.

The only abnormality displayed is hyperinnervation; up to five axons may contribute to the ending (Plate 4.2 B). The average is 2.3 axons per Golgi tendon organ at 6 weeks P.O., 3.4 at 8 weeks, 3.5 at 12 weeks and 2.5 at 30 weeks P.O. However innervation by a single axon is still seen (Plate 4.2 A)

Summary and comment

Large-diameter axons comparable in size to the presumed Ia axons observed in the crush study, are present in spindles from 6 to 30 weeks P.O. However not one example has been noted of a recognisable primary ending.

This observation may have two interpretations: Firstly, none of these large-diameter axons were originally Ia axons, and therefore were not capable of forming a primary ending; or secondly, that after an extended period of denervation, the nature of the intrafusal bundle is changed, either through the prolonged lack of the appropriate innervation, or through incorrect innervation by alpha motoneurons.

If the former explanation is true, then this argues in favour of the specificity of sensory axon function. It does however seem unlikely, though not impossible, that not one Ia axon has returned to a spindle, in which case the latter explanation may hold some validity.

The example shown in Fig. 4.4 gives the impression of repeated attempts at innervation, all of which are rejected. This may be a Ia axon which is returning to the spindle, but is unable to elaborate an ending because of the effects of prolonged denervation.

The restoration of motor terminals to spindles is not impressive, there being relatively few recognisable terminals.

Ultra-terminal sprouting is a phenomenon which was not encountered in the crush study, and was observed to occur from both extrafusal endplates and intrafusal p_1 plates in this study. Extrafusal sprouting can be induced by partial denervation, and its presence in this study, may indicate the relatively poor state of reinnervation.

Axons which enter the spindle and leave it, sometimes making terminations first, are present in all analysable spindles. As was predicted from the crush study, one of these axons is seen to form an extrafusal endplate — a presumed alpha invader.

Table 4.2

Weeks P.O	% Return of Ia axons () sample size	% Return of Ib axons () sample size	Presence of intrafusal motor endings	Presence of extrafusal endings
3	0(14)	0(6)	-	-
4	0(15)	0(6)	-	-
5	0(13)	0(7)	-	-
6	86 (14)	100(6)	-	-
7	100(11)	100(5)	+	+
8	100(11)	100(8)	+	+

Supply of axons to PL muscle following ventral root crush and peroneal nerve crush

SECTION TWO

THE REINNERVATION OF MUSCLE SPINDLES BY SENSORY AXONS IN THE ABSENCE OF MOTOR INNERVATION

Introduction

One of the observations made by Ip, Vrbová and Westbury (1977) was that the restoration of sensory terminals is delayed in the absence of motor innervation.

As the time course for the restoration of sensory and motor terminals has been determined during the crush series of experiments, it was decided that this phenomenon should be investigated.

By crushing the appropriate ventral roots, it was calculated that de-efferentation of peroneus longus could be achieved for 6 weeks. If the peroneal nerve was crushed at the knee, then reinnervation of peroneus longus by sensory nerves could start 10 days later.

Methods

A laminectomy was performed and the ventral roots of $L_6 - S_2$ crushed for one minute with a pair of fine watch-maker's forceps (see Techniques). At the same time, the common peroneal nerve was crushed at the knee using the usual procedure. The animals were killed at 3,4,5,6,7 and 8 weeks after the operation, with an overdose of sodium pentobarbitone. The peroneus longus muscles were removed and processed for the production of teased, silver-stained preparations. The contralateral muscles were used as indicators for the efficacy of the staining. The staining was of a high quality in all cases.

Observations and results

The results are summarised in Table 4.2.

In the crush study it was found that Ia axons start reinnervating spindles after 15 days P.O.

At 2 weeks P.O. in these experiments, no axons are visible either in spindles, Golgi tendon organs or nerve trunks.

After 3 weeks P.O. no axons are visible in spindles or Golgi tendon

organs, but there are some fine axons visible in the nerve trunks (see Plate 4.5.D).

At 4 and 5 weeks P.O. more axons are visible in the nerve trunks. Two spindles receive innervation by fine axons (Plate 4.5. E), but no terminals are apparent. One example of a large-diameter axon ($2.9\text{ }\mu\text{m}$) in a nerve trunk can be seen (Plate 4.5. F).

At 6 weeks P.O. 12 spindles from a sample of 14 receive innervation by presumed Ia axons (average diameter $3.2\text{ }\mu\text{m}$). The form and disposition of the terminals have a very normal appearance. Regular spiralling is present on bag₁, bag₂ and chain fibres with a normal pitch/diameter ratio. The endings are all situated in the usual region, i.e. around the equatorial nucleation. No examples of hyperinnervation can be seen. Secondary terminals have been confidently identified on only two spindles and these are poorly elaborated. The presence of motor innervation in the poles has not been detected. All the Golgi tendon organs are now receiving innervation by Ib axons and most have fully elaborated endings, though in some, the terminals are not complete. After 7 weeks P.O. motor axons are seen both extrafusally, with the presence of extrafusar endplates being apparent, and intrafusally, with p₁ plates and trail-like ramifications being found. Golgi tendon organs now all have an innervation of normal appearance. At 8 weeks P.O. spindles have resumed a normal post-crush appearance, though there are fewer motor axons in the pole than at the equivalent time interval in the crush study.

Summary and comment

Recognisable Ia axons are seen 6 weeks after the operation, whereas after the nerve crush alone, they are first seen within P.L., 15 days after the operation, which implies a delay of 27 days in the return of Ia axons in the absence of efferent innervation. These two observations are however not strictly comparable, as the trauma of the laminectomy may be detrimental to the general state of health of the animal, resulting in a slower rate of reinnervation. It is also possible that during these operations the vascular

supply to the sensory axons may have been impaired, which might also delay the reinnervation rate.

What has been shown is that the elaboration of sensory terminals of a normal appearance, both in muscle spindles and in Golgi tendon organs, occurs in the absence of motor innervation, though the rate of return of sensory axons has been reduced appreciably.

SECTION THREE

THE REINNERVATION OF MUSCLE SPINDLES IN THE ABSENCE OF SENSORY INNERVATION

Introduction

The crush study revealed that a proportion of muscle spindles are innervated by axons which are postulated to be alpha invaders. There was however no proof as to the nature of these axons, though their presence does provide an explanation for some physiological results. It was also hypothesised from this study that spindles may, in the absence of the correct innervation, lose the ability to produce specific site reinnervation.

It was decided to investigate the reinnervation of spindles in the absence of the sensory innervation after ablation of the appropriate dorsal root ganglia. If the hypothesis made on the basis of the crush study, that it is the severance of the endoneurial tubes which produces the presumed alpha invaders, is correct, then, were the peroneal nerve to be severed, production of the alpha invaders would be expected to be maximised, facilitating their analysis. It would also be possible to monitor changes, if any, in the fusimotor innervation in the absence of the sensory innervation.

When this series of experiments was being planned, the results from the cut series of experiments had not been analysed, and on the same rationale as for the cut study, it was decided to leave the cut ends of the nerve unsutured.

In 7 adult cats, average weight 2.3 kg., a laminectomy was performed under the usual conditions (see Techniques), and the dorsal root ganglia of S₆ - L₂ were removed. At the same time, the common peroneal nerve was cut at the knee, at the position described in the previous section. The animals were then killed at 5,6,8,9,10,12 and 13 weeks after the operation, with an overdose of sodium pentobarbitone. The peroneus longus muscles were removed from both sides, and processed for the production of teased, silver-stained preparations (see Techniques). The contralateral muscles were processed in the same receptacles as the muscles from the operated side to check for the efficacy of the staining. In all cases the staining was of a high quality.

Weeks P.O.	Axons visible on spindles	Contralateral staining	Tendon Organs receiving innervation () sample size	No. of spindles examined
5	+	+	0(6)	12
6	+	+	0(5)	10
8	+	+	0(8)	15
9	-	+	0(7)	9
10	-	+	0(7)	14
12	-	+	0(8)	13
13	-	+	0(9)	13

Table 4.3

The return of axons to PL muscle after deafferentation and nerve section

Efficacy of operations

The cut operation is self-evidently totally effective. To check that deafferentation was complete, Golgi tendon organs from the P.L. muscle on the operated side were removed and analysed. No innervation was apparent in the tendon organs examined.

Observations and results

The results from the cut study (see previous section) indicate that leaving the cut ends of the nerve unsutured results in an unreproducible pattern of innervation. It was intended that the cut study would provide the base-line against which the reinnervation in the absence of sensory axons could be assessed, but the variable nature of the reinnervation following this lesion has precluded this possibility.

No large axons are visible on any spindles, nor are any sensory terminals, confirming that the deafferentation had been complete. The pattern of innervation is summarised in Table 4.3.

Axons are present in spindles only during the first three periods of restoration, i.e. 5,6 and 8 weeks, though axons are seen growing in nerve trunks during the other periods of reinnervation (Plate 4.5. B & C)

Where axons are present in spindles they frequently take up longitudinally-oriented courses, without producing recognisable endings.

The motor reinnervation of deafferented spindles resembles the motor reinnervation following cut alone (see Section One), in that it is very sparse. On every spindle which could be analysed, axons are present that enter and leave the spindle, sometimes attempting an ending. Only one example of a presumed p_2 plate has been found (Plate 4.5. A). Three examples of a p_1 plate have been found, and one produces an ultraterminal sprout.

Extrafusar endplates are very scarcely seen, and then only in the first three time intervals. They have poorly developed branches within the sole plate. There is one example of an extrafusar endplate with an ultraterminal sprout.

Summary and comment

The results from this series of experiments are for the most part inconclusive, mainly because of the nature of the cut lesion, but the nature of the cut lesion had not been established when the experiment was designed. There are however certain observations which are worthy of comment. The first is that there is a total absence from all the spindles and tendon organs examined of large-diameter axons. This would confirm the identity of the large-diameter axons observed in the cut series of experiments, as being sensory axons.

The presence of a p_2 plate in a muscle spindle at 8 weeks P.O., shows that such terminals which are formed only on spindles, can be maintained despite the prolonged absence of the Ia axon. The presence of ultraterminal sprouting in both extrafusal endplates and p_1 plates corroborates the observations made on material from the cut study.

No effective comment can be made on the long-term effect of deafferentation, as it is known from the cut study, that the absence of axons from the spindle after 9,10,12 and 13 weeks P.O. may be caused by the cut operation alone.

As the hypothesis for the production of the presumed alpha invaders in the crush study, depends on the diameter of the axons in the muscle nerve, it could be argued that some of these formations might be due to sensory axons growing down the incorrect endoneurial tube.

The fact therefore that axons which show alpha-invader-like configurations, are present in this study, adds corroborative evidence to the postulation that such axons in the crush study were originally alpha motoneurones.

SECTION FOUR: THE REINNERVATION OF MUSCLE SPINDLES AFTER NERVE FREEZE

Introduction

On analysis of the crush material, it was postulated that the injury had caused some rupture of the endoneurial tubes which allowed some motor axons to reinnervate spindles that had previously been exclusively extrafusal in their distribution. The cut injury provided an opportunity for virtually any axon to grow down any endoneurial tube, but the restoration of terminals on muscle spindles following this lesion was poor. It was decided to use a lesion which would maintain the endoneurial tubes of the axons intact so as to provide the ideal reinnervating conditions, and this would indicate whether the observations made on crush material were attributable to endoneurial damage or not.

Mira (1979) claims that freezing nerves produces an interruption of the axon, but leaves the endoneurium intact, allowing axons to regenerate to their original positions.

The act of freezing however does not, per se, produce these conditions. as Takano (1976) used freezing to produce an " alpha-muscle ". a condition also occurring after cut and resection (Thulin, 1960), from which it can be inferred that gamma axons were presented with a gross obstacle to reinnervation after freeze; an observation inconsistent with Mira's findings.

The most probable explanation of this lies in the speed with which the freeze took place. Takano (1976) produced the lesion by the application of dry ice for protracted periods of up to five minutes. This would result in relatively slow cooling and freezing of the nerve. Slow cooling produces large ice crystals which could rupture the endoneurial tubes. Mira (1979), however, used a liquid-nitrogen cryode which was cooled by a pulse of liquid nitrogen introduced under pressure. This produced an almost instantaneous freezing of the nerve. It was decided to emulate Mira's method as closely as possible. His equipment was however unobtainable in this country, so a different freezing technique was devised. (see Techniques)

Methods

The common peroneal nerve was frozen at the knee in six adult cats, (average wt. 2.1 kg.) using the usual operating procedures. The animals were killed after 7,16,28,35,42 and 56 days post operation, by an overdose of sodium pentobarbitone. The peroneus longus muscle was processed for the production of teased, silver-stained preparations of muscle spindles and tendon organs.

Preliminary procedures

It was essential to establish that the denervation produced by this freezing technique was complete. This was achieved in two ways.

One animal was killed seven days after the operation. On examination of the muscle spindles and tendon organs from P.L., no axons could be seen within the intramuscular nerve trunks, or within spindles or tendon organs. Axons in the contralateral muscle processed in the same receptacles were well stained. The animal killed after 16 days P.O. did not have axons present in spindles either, though reinnervating axons were present in intramuscular nerve trunks, again indicating that the denervation produced by the freeze technique was total. The other evidence comes from examining the peroneal nerve distal to the injury. The nerves from the 4 and 6 week P.O. animals were processed for electron microscopy (see Techniques), and an examination revealed that the myelin sheaths of the axons were all noticeably thinner when compared to normal axons. This is a feature of reinnervating axons. For a comparison between the normal and post-freeze sections of the peroneal nerve, see Plate 4.6.

The calculation of reinnervation time for the freeze lesion

Reinnervation time is defined as the number of days that the fastest growing axons are calculated to have entered and been reinnervating a muscle. As some axons were found to be present within the muscle after 16 days, it was decided to calculate the days R.T. by subtracting 16 from the post-operative time in days.

Analysis

The analysis used the same conventions as those adopted for the crush study.



Table 4.4

Supply of Ia axons to spindles after nerve freeze.

Days P.O.	Days R.T.	% Return () sample size	Mean axon diameter (μ m). () no. of measurements	Standard Deviation
7	-	0(14)	-	-
16	-	0(17)	-	-
28	12	100(13)	3.5(30)	0.9
35	19	100(15)	5.4(27)	1.0
42	26	100(13)	5.2(24)	0.7
56	-	81(11)	5.1(18)	0.9

Observations and results

For reasons which will be discussed later, the observations made on the 8 week P.O. material will be presented separately.

1) Restoration of primary endings

The results are summarised in Table 4.4.

a) Rate of return

The first time axons are seen in spindles is at 12 days R.T. At this stage, all of the 13 analysable spindles receive innervation by Ia axons. This is comparable to the time of 10 days R.T. taken by the first wave of Ia axons returning in the crush study. Thereafter, in both 19 and 26 days R.T. material, all spindle capsules receive innervation by Ia axons.

b) Diameter of Ia axons

The diameter of the Ia axons is shown in Table 4.4. It can be seen that, after 12 days R.T., the diameter of the axons is less at $3.5\text{ }\mu\text{m}$, than the final thickness of over $5\text{ }\mu\text{m}$, but it is comparable to the value of $3.0\text{ }\mu\text{m}$, which is the thickness of Ia axons at 10 days R.T. in the crush study.

c) Hyperinnervation

No examples of hyperinnervation have been seen in the freeze material.

d) Distribution of endings to intrafusal fibres

The normal distribution of terminals to the different types of intrafusal fibres is resumed even at 12 days R.T. No examples can be seen where the primary ending does not supply any one kind of intrafusal fibre.

e) Form of the ending

The form of the ending resumes a normal appearance. Regular spirals have formed around the equatorial region and bag_1 and bag_2 fibres have resumed the differences in the nature of their spiralling.

The average pitch/ diameter ratio is 0.3, and no examples of the high value pitch/ diameter ratio spirals which are present in the crush study, can be seen.

f) Position and extent

The primary terminals are invariably restored over the equatorial nucleation, and have an average length of 270 μm , which is close to the normal value of 300 μm . No examples have been seen where the primary terminals extend into the S_1 region.

Table 4.5
Supply of II axons to spindles after nerve freeze.

Days P.O.	Days R.T.	% Return () sample size	Mean axon diameter (μ m) () no. of measurements	Standard Deviation
7	-	0(14)	-	-
16	0	0(17)	-	-
28	12	33(12)	2.1(12)	0.3
35	19	60(15)	3.0(21)	0.8
42	26	69(13)	3.4(27)	0.7
56	-	66(11)	2.0	0.7

2) Restoration of secondary endings

a) Rate of return

The results for the restoration of secondary endings are shown in Table 4.5.

It can be seen that at 12 days R.T., one third of spindles are receiving innervation by group II axons. This is approximately half the level of reinnervation eventually achieved, and indicates that the group II axons are slower in returning than the Ia axons after freeze, as the latter have achieved 100% reinnervation at this stage.

b) Diameter of group II axons

The average diameter of group II axons at 12 days R.T., is 2.1 μm , slightly lower than the reinnervating group II axons after crush at this stage (2.7 μm), and also less than the values after 19 and 26 days R.T. This is consistent with the observation that, at 12 days R.T., group II axons are at an early stage of reinnervation.

c) Hyperinnervation

No examples of hyperinnervated secondary endings have been found.

d) The form of the ending

Secondary endings resume their normal appearance, and both annulospiral and flower-spray terminals are reformed.

In comparison to the crush study, the secondary endings are very well restored.

e) Distribution of the endings

Restored secondary endings are distributed mainly to bag₂ fibres and chain fibres, though a small contribution is also made to bag₁ fibres.

f) Position and extent

Restored secondary terminals are situated in the S₁ position in the majority of cases, though two examples are seen in the S₂ position. They are more extensive than in the crush study, having an average length of 260 μm , though this is still low when compared to the normal range of 250-700 μm .

g) Interactions with other axons

There are no examples of the secondary or primary endings occupying the site of the other, and causing rejection, nor are there any examples of secondary endings sending branches through the equatorial region to form more secondary terminals on the S_1 position on the other side. In fact, they have a totally normal appearance, apart from the diameter of the supplying axon.

Table 4.6

Supply of motor axons to spindles after freeze.

Days P.O.	Days R.T.	Mean diameter of axons supplying p ₁ plates (μm)			Mean diameter of axons supplying p ₂ plates (μm)			Mean diameter of axons supplying trail endings (μm)		
		\bar{x}	n	S.D.	\bar{x}	n	S.D.	\bar{x}	n	S.D.
7	-	-	-	-	-	-	-	-	-	-
16	0	-	-	-	-	-	-	-	-	-
28	12	1.0	3	0.3	1.0	2	0.5	0.7	2	-
35	19	1.4	9	0.2	1.5	4	0.5	1.0	8	0.4
42	26	1.5	8	0.2	1.7	5	0.4	1.1	9	0.3
56	-	1.3	7	0.3	1.4	4	0.4	1.9	6	0.4

The motor innervation

1) Restoration of extrafusal motor endplates and p₁ plates

The results for the restoration of motor endings after nerve freeze are summarised in Table 4. 6.

At 12 days R.T., extrafusal endplates have resumed a normal appearance. No examples of hyperinnervation or abnormality are seen at this stage or later.

Although the extrafusal endplates have resumed a normal appearance at 12 days R.T., out of a sample of 24 poles, only three presumed p₁ sole plates are reinnervated, and these are poorly elaborated. At this stage, the ending usually consists of a single branch within the sole plate. After 19 days R.T, p₁ plates are more commonly seen, with their presence being noted on 9 out of 28 poles.

The endplate now has a more normal appearance with several branches within the sole plate. Examples of the Y-shaped formation can now be seen. (see Plate 4.7.C) At 26 days R.T., they are noted on 8 out of 24 poles.

a) Diameter of axons

The diameter of axons supplying p₁ plates is shown in Table 4.6. It can be seen that the earliest value at 1 μ m is lower than that at 19 and 26 days R.T., indicating that, at 12 days R.T. innervation is in its early stages.

b) Hyperinnervation

No examples of hyperinnervation are seen.

2) Restoration of p_2 plates

The results are summarised in Table 4. 6.

a) Rate of return

Recognisable p_2 plates are not well-elaborated after 12 days R.T., but there are two examples of an early restoration. These are both elongated plate-like endings, of average length $77 \mu\text{m}$, and they are each supplied by a single axon.

At 19 days R.T., the characteristic form of the p_2 endplate has been resumed (see Plate 4. 7 A), and several examples of normal appearance are seen at this stage and at 26 days R.T.

b) Diameter of axons supplying p_2 plates

As is the case with the axons supplying p_1 plates, the diameter after 12 days R.T. is appreciably lower than that at the succeeding time intervals.

At both the subsequent time intervals, the average diameter of the axons supplying p_1 plates is lower than that of those supplying p_2 plates.

c) Distribution of p_2 plates

When the identity of the intrafusal fibre on which the p_2 plates are formed, can be determined, these are seen to be bag_1 fibres. One p_2 plate however is supplied by a branch of an axon, the other branch of which goes on to form another p_2 plate on a different intrafusal fibre to the first. (see Plate 4.7 D)

Examples are also seen, where p_2 plates are supplied by branches of axons, the destination of whose other branch cannot be discerned. (see Plate 4.7 B)

d) Abnormalities in p_2 plates

Apart from the abnormality in distribution noted previously, another abnormality has been observed.

In one example, an ultraterminal sprout is formed (not in itself an abnormality), but this sprout goes on to innervate a presumed p_1 sole plate

situated in a more polar region on the same intrafusal fibre (a bag_1 fibre), and forms an ending with a similar appearance to a p_1 plate. (see Fig. 4.9)

In another example of terminal sprouting, the sprout goes on to form a brush-like terminal, similar to those seen in trail ramifications on an adjacent chain fibre. Examples of terminal sprouting with the sprout ending in the usual vesicular axonal swelling have also been seen.

3) Restoration of trail ramifications

The results are summarised in Table 4.6.

Trail-like ramifications can be seen in the juxta-equatorial region after 12 days R.T., but only two can be confidently identified as a trail ending. After 19 and 26 days R.T., trail endings have a normal position and distribution, i.e. mainly to bag_2 fibres and chains, and they are also normal in appearance.

The diameter of axons supplying trail ramifications follows the pattern for the p_1 and p_2 plates. At 12 days R.T., the diameter of the supplying axons is lower than at subsequent time intervals. At each stage this is lower than that of the axons supplying p_1 and p_2 plates.

4) Visiting axons

Excluding recurrent axons from recognisable trail ramifications, axons which enter the spindle only to leave it again, having made an attempt at an ending have not been observed . Occasionally axons passing through spindles without making endings are present.

Table 4.7

The supply of axons to Golgi tendon organs
after nerve freeze.

Days P.O.	Days R.T.	% Return () sample size	Mean axon diameter (μ m) () sample size	Standard Deviation
7	-	0(6)	-	-
16	0	0(7)	-	-
28	12	100(7)	3.0(9)	0.9
35	19	100(9)	3.6(21)	0.5
42	26	100(9)	4.9(18)	0.6
56	-	100(8)	4.7(18)	0.7

Golgi tendon organs

The results are summarised in Table 4.7.

1) Rate of return

By 12 days R.T., all tendon organs seen have received innervation by Group Ib axons, and this is the situation after all subsequent time intervals.

2) Nature of the endings

All the tendon organs examined have terminals which are of an entirely normal appearance.

3) Diameter of the axons

The diameter of the Group Ib axons increases from the initial low value of 3.0 μm to 4.9 μm at 26 days R.T.

The lower value at 56 days P.O. is assumed to be caused by the increased damage due to the injury, in this one case, and it is also assumed that the axons have been returned for a shorter time than in the 42 day material (P.O.).

4) Abnormalities

The only abnormality in the reinnervation is that of hyperinnervation. This is seen only in the 19 days R.T. material.

There are three examples in all. One is supplied by four very fine axons, and the other two, by three very fine axons. In all cases, these very fine axons contribute towards the terminations which have a normal appearance.

Eight Week Post-Freeze Material

As can be seen from Table 4.4, only 81% of spindles have received reinnervation by Ia axons eight weeks after the freeze operation. This implies that the damage caused by the freeze injury in this one case, has greatly exceeded that caused at the other time intervals, as, even at 12 days R.T., there has been 100% return of Ia axons, and even the diameter of the Ia axons which have returned after 8 weeks, are on average lower than that of those restored at 19 and 26 days R.T. This means that comparison in terms of R.T. cannot be made between the 8 weeks P.O. material and the remainder of the freeze material.

The reason for the lack of return of 20% of the Ia axons is presumably increased endoneurial damage caused by too extensive an area of freezing. The level of damage is comparable to that caused by the crush injury, and may provide additional information to the crush study, as at 56 days P.O., spindles may have been deprived of innervation for longer than spindles during the crush study, where the longest period of denervation confirmed was 48 days.

Sensory innervation

1) Restoration of primary endings

Of the nine analysable capsules which receive innervation by Ia axons, two produce primary terminals which have an entirely normal appearance, distribution and position on the spindle.

There is one example of a single axon entering the equatorial region, branching and terminating in axonal swellings (Plate 4.9 A).

Another spindle has two primary endings, one situated over the site of equatorial nucleation, and the other offset to one side, distributed mainly to a separate bundle, but extending into the S_1 region so that it overlaps a secondary terminal situated on the original intrafusal bundle. (Plate 4.8 A)

In another spindle one branch from the first branching node of the Ia axon extends out into the S_1 region (Plate 4.9 C), where an axonal swelling is made in close opposition to the secondary terminal, and a branch from this returns to the primary region.

One example is found, where a presumed secondary axon approaches the equatorial region from a pole, makes a secondary ending in the S_1 position and one branch then grows through the equatorial region and forms another secondary ending in the other S_1 position. While passing over the area of equatorial nucleation however, it branches and supplies rudimentary terminals, mainly to the chain fibres. (Fig. 4.10)

In one example (Plate 4.9 B), one branch of the Ia axon produces terminals in the juxta-equatorial position.

Another example has been observed, where the primary ending produces only irregular half-ring formations (Plate 4.8 B).

2) Restoration of secondary endings

Apart from the example mentioned above, secondary endings, where present, resume a normal position and distribution to the intrafusal fibres (Plate 4.8 B), although the average diameter of the axons is smaller than for the other periods of the nerve freeze (average diameter $2.0 \mu\text{m}$ in a sample of 18).

Motor innervation

1) Restoration of extrafusal endplates and p_1 plates

All examples of extrafusal endplates have an entirely normal appearance and there are no examples of either hyperinnervation or terminal sprouting.

P_1 plates can be identified in 6 out of a possible 29 poles, and are distributed to bag₁ fibres, when it is possible to identify them. The average diameter of the supplying axons is $1.3 \mu\text{m}$.

The form of the ending is usually normal, but there are two examples, where there is only one branch within the plate. In one example, a "Y"-shaped formation has been formed, and the two plates are distributed to different intrafusal fibres, one of which can be identified as a bag₁ fibre.

2) Restoration of p_2 plates

No p_2 plates can be confidently identified, though there is one example

of an elongated plate-like ending, length 50 μm , in one spindle. This is supplied by a single axon of diameter 1.9 μm , but the intrafusal fibre, on which it is situated can not be identified.

3) Restoration of trail endings

Trail-like ramifications are present on all analysable poles, but confident identification is not possible.

4) Invading axons

In contrast to the previous freeze material, each analysable spindle receives fine axons which perform numerous tortuous convolutions, sometimes producing endings before leaving the spindle. These formations are similar to those seen in the cut and the crush studies.

Restoration of Golgi tendon organs

Six out of a sample of 8 Golgi tendon organs receive innervation by presumed Ib axons. The terminals, where present, have an entirely normal appearance.

Summary and comment

The spindles taken from the 5,6 and 7 week post-freeze muscles, indicate that the freeze lesion has produced the effect that was predicted by Mira (1976), i.e. there has been axonal interruption without endoneurial disruption. This has been inferred from the observation that all spindles receive innervation from Ia axons by the same time interval, i.e. there is no first and second wave of reinnervation. There are also no signs of α innervation, a condition which has been attributed to endoneurial rupture.

Sensory terminals are quickly restored to normality. The motor innervation is slower in returning, but the elaboration of the p_2 plates is complete at much earlier times R.T. than in the crush study. Some abnormalities are apparent in this freeze material.

The example illustrated in Fig. 4.9 shows that the axon which has formed the p_2 plate, is also capable of forming an ending on a p_1 sole plate, or that the beta axon, which is forming the ending on the sole plate, is also capable of forming a p_2 plate, presumably on a p_2 site. This may indicate that the form of the ending which reinnervating axons produce, is influenced by the nature of that which they are innervating.

For reasons explained previously, it was interpreted that the freeze lesion, in the one instance of the 8 weeks P.O. material, caused greater damage than expected, resulting in potentially a longer period of time when the muscle was denervated (denervation time) than had occurred in the crush study.

The fact that a presumed secondary is beginning to form terminals around the chain fibres in the region of equatorial nucleation, and that Ia axons are beginning to form primary endings with branches elaborating terminals in the S_1 position, indicates that the mechanism which causes the site specificity seen in the crush study is no longer operating adequately. This may be a function of the denervation time.

SECTION FIVE

THE REINNERVATION OF MUSCLE SPINDLES AND GOLGI TENDON ORGANS AFTER A MINIMUM PERIOD OF DENERVATION

Introduction

The very poor success rate of restoration of nerve terminals on spindles in the cut series of experiments could have at least two explanations. The first is that very few, if any, of the axons are growing down their original endoneurial tubes, so that a mis-match of axon and intrafusal substrate makes elaboration of terminals impossible. The other is that during the period of denervation, ranging from 6 weeks to 10 weeks in the cut study, the spindles were atrophying or undergoing metabolic change, so that during the early stages of any subsequent innervation there was insufficient site specificity to cause elaboration.

To see if the denervation time does indeed produce an alteration in, for example, the specificity of the reinnervation or the speed with which reinnervation proceeds, two pilot experiments were devised.

The first was to crush the nerve at the muscle entry, which would provide the minimum period of denervation, and the second was to crush the nerve repeatedly, so that reinnervation was prevented for a period of 50 days.

Unfortunately the animal being used for the repeat crush study died following the last crush operation. So only the nerve entry crush study can be presented here.

Method

In a single cat, the peroneal nerve was exposed and the branch supplying peroneus longus was traced, then crushed at its point of entry into the muscle. Three weeks later the muscle was removed and processed for the production of teased, silver-stained preparations.

Observations and results

Sensory restoration

1) Primary restoration

Out of a sample of 17 analysable spindles all receive a Ia axon. There are no examples of hyperinnervated spindles.

a) Form of the ending

In the majority of spindles, the primary ending resumes a very normal appearance, the only sign that they are reinnervated spindles being that the diameter of the Ia axons is below normal and that they are not myelinated. The average diameter of the Ia axons is $4.1 \mu\text{m}$.

There are regular spirals present around the equatorial nucleation, which have a pitch/diameter ratio of 1, close to normal.

The terminals are distributed to bag_1 , bag_2 and chain fibres, (see Plate 4.10 A) and it has often been possible to confirm that the characteristic terminals distributed to bag_1 & bag_2 fibres have been restored.

2) Secondary restoration

In 11 spindles, secondary endings are very well restored, showing both annulospiral and flower-spray endings. (see Plate 4.10 B)

Their extent is entirely that of the normal ending and they are also in the S_1 position. The average diameter of II axons is $3.5 \mu\text{m}$.

Motor restoration

1) Extrafusal endplates and p_1 plates

Extrafusal endplates are restored to a normal appearance. No examples of hyperinnervation or ultraterminal sprouting have been observed.

Intrafusally, p_1 plates are observed on six spindles. All of these p_1 plates have a normal appearance, and where identification of bag fibre type is possible, they are positioned on bag_1 fibres. The average diameter of the supplying axons is $1.1 \mu\text{m}$.

2) Restoration of p₂ plates

There are only two examples of restored p₂ plates. Neither of these are well-restored, with the branches within the plate ending in tapers rather than in the usual swellings.

The average length of the plates is 45 μm , and they are both supplied by thin axons of average diameter 0.9 μm . Identification of the intrafusal fibre types which they supply cannot be made.

3) Restoration of trail endings

Trail-like ramifications are present in all spindles, although the endings are not well-developed which makes the analysis of the distribution of the ramification difficult. Where this is possible, it is mainly the chain fibres that are supplied. The average diameter of axons supplying trail-like ramifications is 1.1 μm .

4) Invading axons

There are no examples of axons which enter the spindle and then leave.

Golgi tendon organs

All the Golgi tendon organs receive innervation by Ib axons. They all produce terminals of normal appearance. The average diameter of the supplying axons is 3.9 μm . There are no examples of hyperinnervation. There are 9 tendon organs in all.

Summary and comment

If the same latent period and rate of regeneration, as calculated from the crush study, apply to these experimental conditions, which does seem likely, then it can be calculated that these muscle spindles and tendon organs are being examined after 15 days R.T. The fact that all the analysable spindles are receiving innervation by this stage, indicates that the endoneurial tubes have remained intact after this crush operation, an observation borne out by

the lack of invading axons. This implies that axons are returning to their original sites of innervation. The restoration of sensory terminals in this study is more advanced than at the equivalent periods R.T. in the crush study.

This might indicate that the speed with which terminals are elaborated depends on the period of time for which the spindles are denervated.

CHAPTER FIVE

CHAPTER FIVE

DISCUSSION

The identification of reinnervating neurones

As mentioned previously the rationale adopted for identifying neurones was that of assuming that axons which formed terminals that were identifiable by their similarity in form and position to normal endings, had been axons which, prior to the lesion, formed the said endings.

This assumption has been made implicitly in the previous studies of spindle reinnervation (Ip, Vrbova & Westbury, 1977 , Barker & Boddy, 1980), and it will now be examined in the light of the findings presented in this study. The diameter of an axon is one parameter which has been used to differentiate between motor and sensory axons at the level of the spindle. This does not seem an unreasonable practice, as it has been shown that, in reinnervated nerve trunks, relative differences in diameter between the different populations of axons are restored (Devor & Govrinn-Lipman, 1979).

Evidence that differences in diameter between motor and sensory axons at the spindle are restored is provided by the crush study, in which the axons supplying the putative primary endings were invariably the largest, as were the secondary endings the second largest. It might however be argued that the diameter of the reinnervated axon, and the terminals it produces, is influenced by the substrate which it innervates, and that the alleged Ia axons were not previously Ia axons at all, but merely being induced to form a primary ending by their presence on the primary site.

Evidence from the de-afferentation and cut studies (Chapter 4, Sections 1 and 3) would suggest that this hypothesis is not valid.

The results from the cut study show that some GTOs are being successfully restored by large-diameter axons and that large-diameter axons are present in the muscle spindles (though not necessarily forming recognisable endings for reasons to be discussed later). The results from the de-afferentation and cut study show that no GTOs are restored nor are there any thick axons within the spindles. If motor axons were capable of forming endings on sensory structures, then it would be expected that tendon organs in the de-afferentation and cut study would have some innervation. As noted previously, this is not the case. The lack of large-diameter axons in the de-afferentation and cut study suggests that large-diameter axons in reinnervated material can be classified as sensory in origin.

These two observations both indicate that the large-diameter axons are sensory in origin, and therefore, that the primaries, secondaries and GTO endings, in short-term reinnervated material, are not formed by way of induction by the substrate, from axons which were previously motor in origin.

Although the putative inductive effect of the primary site does not cause alteration in motor axons, it may be possible that a previously group II sensory axon might be induced to form a primary ending on the primary site and vice versa. This hypothesis is not consistent with the observations made in the crush study (Chapter 3), where it has been seen that, during the period between the first and second " waves " of Ia innervation (to be discussed in greater detail), the region of the primary ending is not receiving any innervation, despite the fact that II endings, tendon organs and the motor supply to the poles are well-restored. This indicates that the region of primary innervation will not, at this stage, accept any other form of innervation other than Ia. In fact, there is evidence, provided by observations that

fine axons entering the equatorial region terminate in spherical lozenges, that attempts at innervation are being rejected. The fact that a secondary terminal sends a branch across a vacant equatorial region, without attempting to form an ending on that site, is further evidence that only Ia axons are capable, at that stage of reinnervation time, of forming terminals on a primary site.

The possibility of a previously Ia axon being induced to produce a group II ending by a vacant secondary site (possibly by sprouting, which will be discussed later), is inconsistent with observations made in the crush study.

It was shown in Chapter 3 that the first " wave " of Ia innervation occurs earlier than the innervation by group II axons e.g. at 11 days R.T., 75% return for Ia axons, 18% for group II axons, allowing ample opportunity for Ia axons to innervate and elaborate on a secondary site. No examples of this were seen.

It could be argued that Ib axons might form endings on sensory sites within the spindle and vice versa, but, if the muscle spindle is capable of discriminating between two forms of ending which can have a very similar form, and occur on the same intrafusal bundle, separated only by 10s of microns, i.e. primary and secondary, then it seems most unlikely that an axon which normally forms an ending of different form on a different substrate, and which normally responds to the opposite physiological stimulus, would be capable of forming an ending on a primary site, and for the same reasons, nor would a Ia or a secondary on a GTO. This is consistent with the observations made in the cut study which showed that, although the terminals on some GTOs were restored, there were none restored on primary sites.

The evidence presented thus far indicates that in reinnervated

muscle spindles, after short periods of denervation, sensory axons are distinguishable from motor axons by virtue of their diameter, and that Ia axons alone re-establish primary endings, and that II axons alone re-establish secondary endings, and there is also an indication that group Ib axons and motor axons are not capable of forming primary endings. This evidence suggests that the assumption is correct that axons which formed terminals that were recognisable by their similarity in form and position to normal endings, had been endings which, prior to the lesion, formed the said endings.

The fact that a paciniform corpuscle and a GTO share the same axon does not necessarily invalidate the hypothesis proposed. No endings are elaborated within the corpuscle, so any branch which grows into the corpuscle will appear to innervate it, but in fact may have no functional significance.

Specificity of sensory reinnervation

The sensory reinnervation of muscle spindles will now be discussed with regard to specificity. To facilitate this, different degrees of specificity will first be defined. The most precise form is "original terminal" specificity, i.e. the reinnervating axon is capable of restoring a terminal only on the exact site that it had been innervating prior to the lesion. A less precise form is "site-type" specificity, i.e. the reinnervating axon is capable of restoring a terminal only on an exact site where an axon of the same type had previously innervated. For example, a Ia axon is capable of innervating any denervated spindle, but only on the primary site.

It is difficult to prove "original terminal" specificity, as, in the crush study, the majority of axons are returning, by virtue of their intact endoneurial tube, to the same spindle anyway, and there

is no means of knowing whether the escaped axons have returned to their original spindles or not. The fact however that 100% reinnervation is achieved in a wave, rather than gradually, would militate against " original terminal " specificity, as it would seem unlikely , though not impossible, that the entire population of " escaped " axons could find their target spindle within such a relatively short time.

The fact that, in the cut study, there are no well-restored primaries, does not necessarily argue in favour of " original terminal " specificity, as time dependent factors in the mechanism of ending elaboration, may be operating (discussed later). The fact however that GTOs do produce reasonable endings ^{argues} against " original terminal " specificity, as, given the circumstances through which the regenerating axons have to grow, it would seem most unlikely that many GTOs could receive back their original axon.

If comparison is made with other regenerating afferent axons, then it has been shown in the cat by Horch & Burgess (1980) that the axons are not specific to their original site of innervation. Their observation is consistent with the hypothesis produced here. No mention is made in the other studies of spindle reinnervation, of this form of specificity.

The other form of specificity, that of " site-type ", will also be discussed in relation to the sensory nerves.

As has been shown in Chapter 3, primary (and secondary) endings are formed in their normal positions. It could be argued that the presence of primary terminals does not in itself indicate " site-type " specificity, but shows that, as Ia axons are regenerating down their original endoneurial tubes, then the first intrafusal structure they encounter is the site where the normal ending is formed, and the ending

is in fact elaborated there merely because it is the site of the first encounter. This does not exclude the possibility that an ending could be elaborated elsewhere on the bundle if the opportunity arose. There are two indications that this is not the case. One is provided by the secondary sensory innervation. This is also restored to its original position and extent, but formations are seen where a branch of a secondary axon traverses the area of equatorial nucleation to make another secondary ending at the opposite side of the equatorial region, without any attempt being made to elaborate terminals in the primary region.

This suggests, as mentioned previously, that either the group II axon is not capable of forming endings at the primary site, or that the region of the primary ending does not provide any signal to the nerve for the ending to elaborate, or that both occur. Findings from 8 week freeze material suggest that group II axons are in fact ultimately capable of forming endings on the primary site (after the spindle has been denervated by group Ia axons for long periods). This observation implies that the substrate of innervation is causing " site-type " specificity of innervation, after short periods of denervation, only allowing Ia axons to elaborate on primary sites. The same of course applies to group II axons.

The other indication that the observed correct reinnervation by Ia axons is not just the effect of proximity of the correct part of the intrafusal bundle to the end of the endoneurial tube, is that the second " wave " of Ia innervation (Chapter 3, page 67) is also restored to the normal position on the intrafusal bundle. The hypothesis formed to explain this observation suggested that the damage to approximately 25% of the population of the Ia axons was significantly greater

than to the rest. All the myelinated axons in the nerve trunk have been shown to be damaged at the site of the lesion, so this probably indicates that the extra damage causing the delay, is to the supporting tissues within the nerve trunk, as it is known that the presence of such tissues is of great importance in the successful regeneration of nerves (McMahan, Edgington & Kuffler, 1980).

It has been shown by Strain & Olson (1975) that Laplace's law can be applied to compression phenomena within nerve trunks. This states that the tension in the wall of a cylinder is proportional to the difference between internal and external pressures and the radius of the cylinder ($T = P \times R$).

It is therefore more likely that for any applied pressure, the endoneurial tubes of the larger axons, i.e. the Ia axons and the alpha motoneurones, will be ruptured. Once ruptured, the opportunity will occur for the axons to grow either down adjacent endoneurial tubes or outside a pre-existing route altogether. That these both happen has been shown by Haftek & Thomas (1968), in that they showed the merging of adjacent endoneurial tubes at the site of the lesion and " escaped " axons (Holmes & Young, 1942) growing outside endoneurial tubes. These " escaped " axons would necessarily take longer to return to the spindle and could account for the second "wave" of Ia innervation. The fact that all spindles in the crush study receive Ia innervation indicates that even when Ia axons do not have physical guidance, they will still only form endings over the usual position. Hence the evidence provided by the crush study is that there is " site-type " specificity for the position of the primary ending.

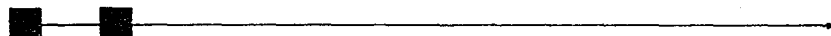
The evidence presented so far indicates that " site-type " specificity occurs for sensory axons. As the Ia axon is known to induce the

formation of muscle spindles in development (Zelena, 1964, Zelena & Soukoup, 1973, Milburn, 1973), it does not seem unreasonable that the prolonged absence of a Ia axon may result in such effects as the diminution in "site-type" specificity. In the crush study, the intrafusal bundle has been left without the influence of the Ia axon for a maximum of 48 days, and although " site-type " specificity is very marked even after this period of denervation, there is one indication that this form of specificity is beginning to break down. This is the example where a secondary ending in an S_1 position, is situated more equatorially than usual, to the extent that a presumed Ia axon within the periaxial space does not make a primary ending.

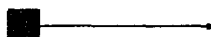
As was discussed in Chapter 4, the exact length of denervation time in the cut study was impossible to determine, but the results indicate that any spindles receiving innervation would have been denervated for at least 35 days, and possibly up to 70 days (see Table 4.1). No primary endings of a recognisable form were found in this study, the best attempts being half-ring structures (Plate 4.1 A & B). One explanation of this is that no Ia axons regenerated into any of the spindles examined. This explanation seems unlikely as the crush study indicates that Ia axons are capable of finding their target sites of innervation (as are the extrafusal (McMahan, Edgington & Kuffler, 1980), and in the cut study itself, Golgi tendon organs, extrafusal motor endplates and intrafusal motor endplates are successfully innervated. It is more likely that at least some of the large-diameter axons seen within the spindle capsules are Ia axons which are not forming endings. The fact that Ib axons can produce endings of normal appearance after the same periods of denervation suggests that the lack of primary endings is not due to a failure in the ability of the Ia axon to elaborate

Denervation time in days.

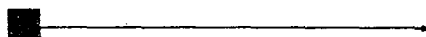
0 5 10 15 20 25 30 35 40 45 50 55 60



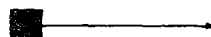
Decrease in rate of thickening of group II axons



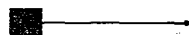
First indication of " site-type " specificity breakdown in crush material



Elaboration of primary terminals losing normal appearance in cut study



Elaboration of primary terminal losing normal appearance in crush study



Rejection of a presumed primary in cut study



Form of a primary terminal losing normal appearance in 8 week freeze study



" Site-type " specificity breaking down in 8 week freeze material

Table 5.1

Table 5.2

Experiment	Days R.T.	Days D.T.	Diameter of Ia axons (μm)	Diameter of II axons (μm)	Source
Nerve entry crush	15	6	4.1	3.5	Chapter 4, Section 5
Nerve crush	15	10	4.1	2.9	Chapter 3
Nerve freeze	15	16	4.3	2.5	Tables 4.4 & 4.5

Table 5.2

Maturation of sensory axons after nerve lesion

an ending, but rather to the intrafusal bundle losing its ability to interact with a Ia axon to produce a primary ending. This could be due to the prolonged absence of Ia axons, or to the innervation of the intrafusal bundle by inappropriate motor axons, or to a combination of the two.

If a spindle can lose the ability to induce a Ia axon to form a primary ending with time, then the interim period between total " site-type " specificity and rejection, may be indicated by a breakdown in " site-type " specificity, and by an increase in the time which it takes a primary ending to elaborate, an index of which may be the size of the Ia axon for a given period R.T.

The sequence of events showing the effect of increasing denervation time is shown in Table 5.1, and this will be explained in the following paragraphs. It would appear , from the data presented in Table 5.2, that over the period from 6 to 16 days denervation time, the primary takes no longer to establish, as judged by thickness of the axon, but the group II axons do appear to be taking longer with increasing denervation time. This is the first indication of a breakdown of specificity occurring after only 10 days R.T.

The first example of a breakdown in " site-type " specificity is shown in the crush material, and this spindle could have been denervated for a maximum of 48 days.

The cut material could have been denervated for a period of from 35 days to 70 days. The 6 week P.O. material (probably 35 days D.T.) only had two large-diameter axons within the spindles: one was producing longitudinally-oriented tapers over the primary site, and the other was making no attempt at an ending and was probably a Ib axon. At 8 weeks P.O., however (approximately 49 days D.T.) two examples of rejected Ia

axons are seen, i.e. some branches from a large-diameter axon, which produces longitudinally-oriented terminals over the primary region, grow out of the spindle. These are seen at all subsequent time intervals in the cut series of experiments until at 30 weeks P.O., a large-diameter axon can be seen to have all its attempts at reinnervation rejected (Fig. 4.4.). The 8 week freeze material could have been denervated for up to 56 days, and there are examples indicating that " site-type " specificity has broken down. In one example, the Ia axon is forming an ending in such a way that it overlaps into a secondary site, and in another spindle, the primary ending is being formed juxtaequatorially.

There are other indications from this material that " site-type " specificity is breaking down: a presumed secondary axon forms some endings over the equatorial nucleation between the two secondary endings which it also elaborates. In another example, branches of a Ia axon form terminals in the juxtaequatorial position, and in another spindle the terminals of the primary consist of irregular half-ring formations. The lack of the normal form of endings could be another indication of the loss of " site-type " specificity. This occurs in crush material after 48 days R.T.

To recapitulate: the observations from the studies reported here indicate that " original-terminal " specificity does not occur in the sensory reinnervation of spindles, and this is consistent with the observations made on other regenerating sensory nerves (Horch & Burgess, 1980).

A very precise " site-type " specificity does however appear to be operating after short periods of denervation. This " site-type " specificity seems to break down gradually over a period until at times after 50 days R.T., reinnervating Ia axons are no longer induced to form primary

endings of a normal appearance.

The fact that the lack of elaboration of primary endings may be occurring at its earliest in the cut study, might indicate that the process of " site-type " specificity is breaking down more quickly under the influence of alpha invaders.

The lack of " original-terminal " specificity and the presence of a precise " site-type " specificity would seem to be the most effective arrangement for ensuring the most rapid functional recovery after nerve injury.

It may be that the trophic effect of the Ia axon may be eventually the cause of changes in the spindles that have rejected them, or have only allowed poor elaboration, so that a " normal " primary ending may be elaborated. This process may be occurring in Plate 4.1 A&B.

Sensory nerve sprouting

Two forms of sprouting could occur in regenerated sensory nerves: one at or near the site of the injury, the other at or near the terminal. Sprouts occurring at the injury site have been shown to persist for long periods (Devor & Govrin-Lipman, 1979), and may account for some of the second " wave " of innervation of Ia axons, so that more than one spindle may be innervated by one Ia axon (a situation known to exist in cat jaw muscles (Kato, Kawamura, & Morimoto, 1982)). Sprouts which grow down endoneurial tubes, if they persist, might give rise to double primaries.

Sensory nerve sprouting at or near the terminal has been observed in cutaneous nerves, and it is possible that such a phenomenon may occur in regenerated Ia, Ib and group II axons. As there are strong indications that in the early stages of denervation, " site-type " specificity is highly precise, sprouting of sensory nerves intrafusally would not be expected and no such examples are seen. In the 8 week freeze material

however there are examples of what could be interpreted as sensory nerve sprouting. Plate 4.9 B shows a nodal sprout onto an area adjacent to the primary, which forms an ending supplied to all the parts of the intrafusal bundle. It might be argued that the absence of the secondary ending is causing sprouting to occur in the primary ending.

Another example is seen in Plate 4,9 C. One explanation is that after a prolonged period of denervation, the primary arrives back, elaborates and then produces a sprout onto an adjacent vacant secondary site. The group II axon subsequently arrives and elaborates causing the Ia sprout to abort its attempt at innervation. Thus sprouting, if it occurs, may be resorbed, as has been shown to happen in cutaneous nerves (Jackson & Diamond, 1981).

Specificity of motor reinnervation

The motor reinnervation of spindles will now be discussed. The results will be examined to see if indications are present to show that alpha, beta and gamma motoneurons are capable of forming any other than their original form of ending, and whether these endings are formed on their original substrate.

It has been established for some time that alpha motoneurons are capable of forming endplates on extrafusal muscle fibres which they did not originally innervate, and which were of a different histochemical type (Karpati & Engel, 1968). It is also known from the studies of McMahan, Edgington & Kuffler (1980) that extrafusal muscle fibres show a marked site specificity , i.e. motor axons reinnervate extrafusal muscle fibres preferentially at the sole plates left by the previous innervation. The muscle spindle might therefore appear to provide reinnervating motor axons with many opportunities for synapse formation, as in the normal state, it has three types of motor ending, and the intrafusal muscle fibres receive

multiterminal and polyneuronal innervation. If the endoneurial tubes have remained intact during the crush operation, and the information from the sensory study indicated that the majority of them have, then it might be expected that the majority of reinnervated motor endings would have a normal appearance, as they were being innervated by their original axons. This was observed to be the case, the main abnormality being hyperinnervation probably caused by sprouting at the site of the injury. These sprouts may not be eliminated and may serve only to decrease marginally the conduction velocity of the innervating axon.

There are however examples of axons in the crush study that, for reasons outlined previously, were considered to have been alpha motor-neurones prior to the crush (" alpha invaders "). Having entered a spindle they may have been induced to branch by the influence of adjacent non-innervated sites of former intrafusal innervation. If these axons form functional terminals at these sites, or even if they form synapses randomly over the bundle, then the effect would be to increase the number of beta axons with static effects on the response of the primary ending (Barker & Boddy, 1980). This interpretation of these observations suggests that there is no form of specificity of innervation operating within a spindle which excludes previously alpha motoneurones from forming functional connections.

There are observations which indicate that any motor axon may elaborate endings on fusimotor sites, and that the ending they elaborate may be influenced, not only by the original function of the axon, but also by the nature of the site it is now innervating.

Plate 3.24 shows an example where five axons made p_1 or extrafusal plate-like endings on a previously p_2 site.

The form of an ending being influenced by the substrate is shown in examples from the freeze study. Fig. 4.9 shows an axon forming a normal p_2 plate, with an ultra-terminal sprout which goes on to elaborate a p_1 -type endplate on a prominent sole plate. Another example is present where a p_2 plate produces a sprout which then ends in a brush-like terminal characteristic of the trail innervation. Such observations imply that the nature of the terminal in the motor innervation may not be a reliable means of identification.

The only observation which could be interpreted as indicating specificity is the frequent presence of terminal swellings, which might be considered to be axons being rejected by the substrate. There are however other explanations that do not require a mechanism of specificity to operate: they may be caused by sensory axons or by the resorption of supernumerary sprouts from motor axons, or they may be part of the normal turnover of endplates as described by Barker & Ip (1966). Hence the evidence provided by this study does not indicate any specificity of motor axon innervation, in fact the results point to a non-specificity of reinnervation of motor axons to muscle spindles. The apparent specificity of motor innervation in the crush study of Brown & Butler (1976) is probably a function of the microarchitecture of the supporting tissues, and not of any inherent specificity of the axons themselves.

Motor nerve sprouting

The phenomenon of motor nerve sprouting has recently been reviewed by Brown, Holland & Hopkins (1981). It can take two forms: that of " nodal sprouting ", where collateral sprouts are formed at a node of Ranvier, and " terminal sprouting ", where outgrowths occur from the nerve terminals themselves. This is observed in partially denervated muscle

and both types of sprout are observed to innervate denervated muscle fibres and to become myelinated.

Nodal sprouting is thought to be induced by the presence of a denervated endplate connected via a vacant perineurial sheath to a node of Ranvier on an intact axon. It is reasoned that muscle in a denervated-like state in some way stimulates sprout growth.

Although this work is not primarily concerned with extrafusal endplates, it is relevant to note that terminal sprouting of reinnervated extrafusal motor endplates has been observed in both the experiments involving the sectioning of the peroneal nerve.

Terminal sprouting is frequently discussed in the context of partial denervation experiments, wherein it is the remaining intact nerves which are induced to sprout onto adjacent denervated fibres. In the nerve section experiments, different conditions obtain, in that all the muscle fibres are denervated. The nature of the injury however was such that, judging by the success of restoration of sensory endings, relatively few axons were reinnervating the muscle. When these few axons succeeded in reinnervating the extrafusal muscle fibres, then conditions of partial denervation would be simulated and both nodal and terminal sprouting might occur, although only examples of terminal sprouting could be confidently identified.

In the remaining experiments of this study, reinnervation was more successful, and no examples of terminal sprouting were seen. With the presence of numerous sites of previous innervation on the intrafusal bundle, it might be expected that sprouting of both types could be present.

In the crush study, Plate 3.23 F shows a p_2 plate with a contribution being made by a nodal sprout. In the extrafusal nodal sprouting, it appears to be the proximity of a vacant endplate on an adjacent fibre

which stimulates sprouting. The fact that this sprout is innervating the same terminal as its parent axon, may imply that the longer p_1 plate has emitted sufficient of the putative stimulus to induce nodal sprouting, before the elaborating terminal either prevents any further release, or absorbs the released substance(s).

It is possible that sprouts produced in this manner may be retracted, and this could account for some of the vesicular axonal swellings seen in the intrafusal motor terminals. Terminal sprouting onto adjacent intrafusal fibres by axons which supply p_1 plates is seen in the cut material (Plate 4.4 D), and this would imply a paucity of denervation on the adjacent intrafusal muscle fibres, which is consistent with the observations made both extrafusally and intrafusally.

It is possible, as discussed previously, that new intrafusal muscle fibres may be formed following denervation. These may also account for the presence of p_1 sprouting, as the "Y"-shaped formation of p_1 plates on different intrafusal fibres may be the result of nodal sprouting.

The freeze material has axons which form p_2 plates, giving rise to ultra-terminal sprouts which go on to innervate vacant sites. This would indicate the ability of the intrafusal muscle fibre to have multi-terminal innervation, so, in the case of muscle spindles, it is the presence of denervated sites of innervation rather than denervated muscle in itself, that is the stimulus to sprouting. This is consistent with the normal inability of the spindle to rid itself of the multiterminal innervation, in contrast to the extrafusal muscle fibre which can do so. This mechanism would ensure that the denervated spindles receive motor innervation, but the fact that axons are capable of forming more than one type of motor terminal would indicate a lack of " site-type " specificity in the intrafusal motor innervation.

Mechanisms of specificity

Work on regenerating motor axons following nerve section in the frog (Letinsky, Fishbeck & McMahan, 1976, McMahan, Edgington and Kuffler, 1980), has shown that regenerating axons innervate extrafusal muscle fibres at previous sites of innervation, and that this is due to regional properties of the remaining basal lamina surrounding the muscle fibres. The basal lamina is known to persist after denervation (Sanes, Marshall & McMahan, 1978). In the normal animal, the basal lamina runs between the muscle fibre and the motor nerve in the synaptic cleft, but over the sensory terminals, and is continuous with the basal lamina in the endoneurial tubes (Merillees, 1960, Barker, Stacey & Adal, 1970, Banker & Girvin, 1971, Adal, 1969).

If an ideal type II injury were performed (Sunderland, 1978), where endoneurial tubes and presumably their basal laminae remain intact, and axons were allowed to reinnervate with the minimum delay, then the rapid resumption of a near normal innervation (Chapter 4, Section) could be explained purely in terms of the physical guidance provided by the basal lamina, and persisting regional differences within the basal lamina.

It has been reported that the basal lamina can prevent sensory axons from making contact with myotubes (Zelena & Sobotkova, 1971) and a shrinking denervated intrafusal fibre (Schröder, Kemme & Scholz, 1979) with the consequent rearrangement of the basal lamina (Sanes et al., 1978) could prevent a regenerating sensory axon from elaborating an ending, and this may be one explanation for the rejected afferents.

While being an important factor, the presence of the basal lamina does not explain all the phenomena of reinnervation, in this or other studies. For example, motor endplates may be formed at other than the

original site (Saito & Zacks, 1969, Frank et al., 1975); collateral and terminal sprouting of motor nerves can occur (Brown et al., 1981); and sprouting of sensory nerves (Diamond, Cooper, Turner & McIntyre, 1976): none of these phenomena could occur if the infilling of a basal lamina skeleton was the only factor which influenced the form of the reinnervation.

It has been noted that secondary terminals can send axons across the equatorial region, without the presence of a primary terminal, to form endings on the other S_1 region. This might be explained by the diminution of the intrafusal fibre which allows the sprouting axon to grow underneath the basal lamina. In the crush study, no terminals were formed by these axons over the primary region, despite the fact that there was no physical barrier. In the 8 week post-freeze material such an axon does produce terminals over the primary region. This would indicate that sensory "site-type" specificity is mediated by factors other than the basal lamina, possibly by trophic influences from the intrafusal fibre.

The sensory sprouting in Plate 4.9 is another formation which cannot be explained in terms of the basal lamina, but rather as sprouting induced by an unoccupied site in the Ia site. It has been shown that, in the absence of the Ia axon, regional histochemical differences diminish (de Reuck et al., 1973) and such factors which cause "site-type" specificity may also diminish.

It is also known that reinnervating alpha motoneurons can alter the histochemical type of the extrafusal muscle fibre they innervate, and therefore presumed alpha invaders may cause a more rapid loss of regional specificities, as noted previously.

Intrafusal fibre splitting

The technique used for staining and analysing spindles in this study does not allow a complete analysis of the intrafusal bundle to be made on many muscle spindles. There are however indications that an increase in bag₁ fibres is occurring after denervation, and this increase in the number of IMFs may well be the explanation for some of the phenomena seen in reinnervated spindles.

An example of such an indication is shown in Plate 3.6, where the primary ending is supplied to a bag₂ fibre, a bag₁ fibre and several chain fibres. There is however another bag₁ fibre which does not receive any contribution from the ending. The lack of innervation points to this fibre being formed after the nerve lesion, as, whether it was created from the splitting of a parent bag₁ fibre, or formed by the maturation of satellite cells, there would be no pre-existing pathway down which a regenerating Ia axon could grow to elaborate a new ending, thus leaving this new fibre without primary innervation during the early stages of reinnervation. The absence of innervation on this fibre would be unlikely to make any difference to the primaries' responses, as the original bag₁ fibre has received its innervation.

Such supernumerary bag₁ fibres which do not receive innervation are present at periods up to 53 days R.T. in the crush study. It cannot be concluded from this that the new fibres do not receive innervation for some time, as it may well be that bag₁ fibres continue to proliferate after 3 months.

Secondary terminals do not reveal the presence of supernumerary bag₁ fibres, as they only make a small contribution, if any, to bag₁ fibres. The motor innervation does however reveal certain features which may indicate the presence of splitting intrafusal fibres. One is the abnormality

of p_1 innervation when the "Y"-shaped formation occurs.

In normal cat hindlimb muscle spindles the two plates making up the "Y"-shape are invariably situated on the same IMF (Barker et al., 1970). In the crush study, there are three examples where the two p_1 plates making up the configuration are distributed to separate IMFs (e.g. Plate 3.17 F). This may have two explanations: The original fibre, probably a bag_1 fibre, as most p_1 plates are distributed to bag_1 fibres, has split so as to produce two fibres. If the original fibre was innervated by a "Y"-shaped p_1 configuration, and the split caused the original sole plates to be separated onto the different fibres, then the reinnervating beta axon would resume its original configuration, but would now be innervating two separate fibres. However, it seems unlikely that the split in bag_1 fibres is going to separate the two sole plates on many occasions, as they are usually positioned parallel to the longitudinal axis of the IMF. This may account for the small number of observations. Another explanation is that the new bag_1 fibre is formed by the maturation of satellite cells. This fibre would not have any innervation. It has been shown that partial denervation of extrafusal muscle fibres can produce sprouting, both terminal and nodal, in the remaining nerves. The situation in a muscle spindle with a new intrafusal fibre is similar in that there is a muscle fibre not receiving innervation, adjacent to one which does. If the new fibre had been formed "de novo" then it would be expected that terminal sprouting would occur. This was not observed in the crush study.

Nodal sprouting is however seen where a denervated endplate is close to an intact nerve, and where there is a pathway between the nerve and the endplate. The "Y"-shaped configuration, previously discussed, is probably formed by nodal sprouting. This would argue in favour of bag_1 fibre split-

ting (Kucera, 1977a) as the mechanism of IMF increase, as a denervated endplate may be present on the "new" fibre, initiating the nodal sprouting.

In a recent study of the effects of denervation on rat muscle spindles (F. Diwan, personal communication), it has been found that supernumerary IMFs were present after the shortest periods of denervation that were investigated (21 days). In contrast to the findings of Kucera, all three types of IMF have been found to increase in number either by splitting or by being formed "de novo".

The evidence presented here indicates that bag₁ fibre splitting is the most likely cause of the formations which have been described in Chapters 3 & 4 of this study. However, this does not imply that an increase in the number of bag₂ or chain fibres does not occur in cat muscle spindles, merely that no evidence for this phenomenon has been provided by this study.

To summarise: it has been shown from work on the rat that IMF increase occurs both during denervation and reinnervation (see Chapter 1). It is likely, that such effects could occur in the cat, and that it is brought about, in at least some instances, by bag₁ fibre splitting.

Growth rates of axons

The observations made during the crush study (Chapter 3) show that the group Ia sensory axons are slower in returning to the spindle than the motor component. This agrees with the observations made by Huber (1900), Ip et al., (1973) and Brown & Butler (1976).

One interpretation of this is that the growth rate of sensory nerves is slower than that of motor axons.

However, previous work (Gutman et al., 1941) did not indicate that there was a significant difference between the growth rates. A more likely

explanation is that, as the crush lesion causes greater disruption to the larger axons, the latent period will be longer and the site of the lesion which they have to cross, will be more disorganised. The smaller motor axons will therefore have a "head" start over the Ia axons, and this may be reflected in the early stages of reinnervation. The fact that group II axons return slightly less quickly than group Ia axons may indicate that the rate of growth of an axon is related to the axon diameter, and that this observation is obscured in this particular study by the differential nature of the lesion.

The rate of growth of axons in the two studies presented here which used nerve section, is impossible to assess due to the highly variable results. This was presumably because of the success rate of axons crossing the site of the lesion. Gutman et al. (1941) showed that the latent period following a cut and suture operation in rabbit, did vary with " closeness of apposition " of the two stumps after the operation. However, both sensory and motor axons did succeed in reinnervating spindles following nerve section. Tello's (1907) observation that " the motor arborisation is well-developed while sensory branches are still growing under the capsule " cannot now be taken as an indication that sensory reinnervation proceeds more slowly than motor, as his description of the sensory ending could be that of any Ia axon reaching the spindle after approximately 50 days of denervation (assuming this figure applies to rabbits).

The results of these studies do however agree with the observation that the recovery was more rapid and complete for both afferent and efferent axons after crush than after section (Brown & Butler, 1976).

The apparent retardation in the growth rate of the sensory axons in the ventral root crush and crush study (Chapter 4, Section 3) could be explained by the blood supply to the nerve trunk being damaged by the vent-

ral root crush, or by the effect of the operation on the health of the animal, or by both. It is known that the endoneurial blood vessels of rodents become permeable to proteins in the presence of regenerating axons (Sparrow & Kiernan, 1981), and damage to the blood supply has been suggested as the cause of different rates of growth in regenerating nerves (Gutman et al., 1941).

No conclusion can however be drawn from the studies reported in this work, as to whether the ability of axons to cross cut and resection or cut and suture, is a function of axon diameter, as these conditions were not tried experimentally.

Nerve freezing has been shown to have varying effects on the different size of axons. The nature of nerve freeze in this study was intended to be as swift as possible, so that endoneurial damage would be minimised. In all but one animal. the lesion on which was unintentionally slightly more extensive than the rest, this was perceived to be the case, as indicated by the relatively well-formed terminals. The slightly longer denervation time, allowing metabolic changes, in conjunction with intact endoneurial tubes, may account for the presence of p₂ plates forming sprouts which innervate other presumed previous motor sites: a phenomenon not seen in the other parts of the study. This observation may point to some specificity of motor innervation which is breaking down.

The lack of presumed alpha invaders or presumed afferent invaders is also an indication that the freeze produced the intended lesion. The results from the animal in 8 week freeze material also unintentionally confirmed that the rate of freezing of nerves does alter the nature of the damage to the nerve trunk.

There is only one observation in these studies which indicates that the growth rate of axons may be a function of their diameter; this is

the relative slowness of group II reinnervation in the crush study. What has been confirmed is that the nature of the lesion is a key factor in determining the success of restoration of muscle spindles.

Reinnervating axons

Hyperinnervation has been seen commonly in reinnervated material, and there is no indication from this study that many of these sprouts are being resorbed during the time course of the experiments. It has been known that nerve lesions can cause axons to produce up to six sprouts, but it has been demonstrated that on average only one sprout is maintained after the lesion (Devor & Govrin-Lippmann. 1979).

Extrafusal muscle fibres are capable of ridding themselves of hyperinnervation both during development (Redfern, 1970 and Bagust, Lewis & Westerman, 1973) and after nerve lesions (McArdle, 1975), and no examples of extrafusal hyperinnervation were seen in this study.

Muscle spindles even in their normal state, do support polyneuronal multiple innervation, so the maintenance of the hyperinnervation caused by nerve lesions is not surprising. The finding that only one sprout is maintained after nerve crush, but that hyperinnervation in the poles is so marked, indicates that much of the phenomenon is due to intramuscular branching.

The diameter of identified reinnervating axons has been shown to be very small while growing (as indicated by the very small diameters of Ia axons in the early stages of reinnervation), but to thicken considerably once the terminals are restored (Fig. 3.1 and Table 3.1). This agrees with the observations of Sanders & Whitteridge (1946) and those of Aitken, Sharman & Young (1947).

For the periods of time over which this study took place, the regenerated axon diameters were always lower than the normal levels. This is

consistent with the results of Gutmann & Sanders (1943) who did not find a return to normal diameters in the peripheral stump until 300 days P.O. The studies of Ip, Vrbova & Westbury (1977) and of Brown & Butler (1976) produced results which are not incocsistent with this finding, in that they record a drop in the conduction velocity of the axons supplying the intrafusal bundle.

Scott (1982) has confirmed that it is distal to the crush site that the greatest reduction in CV takes place (this agrees with the results of Craggs & Thomas (1961, 1964)).

No values for the rate of growth of axons following nerve crush lesions in the cat were found in the literature. The rate calculated in this study of 3.2 mm per day was consistent with the histological observations.

Previous estimates of regeneration rates following crush lesions have been made for the rat and the rabbit, and the rates do depend on how they are measured. The values for rabbits range from 2.5 mm per day (Gutmann et al., 1942) to 4.36 mm per day, and for rats, from 3.0 mm per day for the majority of axons (Forman & Berenberg, 1978) to 4.5 mm per day (Berenberg, Forman, Wood, De Silva & Bemaree, 1977). The value of 3.2 mm per day found in this study does not therefore seem unreasonable.

Values for the latent period following crush lesion range from 5.23 days to 21.6 days, in the rabbit (Gutmann et al., 1942) and from 1.45 days to 3.2 days, in the rat (Forman et al., 1978). The value calculated in this study of 5.8 days does not therefore seem improbable.

Reinnervated spindle morphology

The method adopted for teasing spindles frequently resulted in the most distal part of the poles being damaged. This precluded making observ-

ations as to the length of reinnervated spindles, so no comment can be passed on the observation by Arendt & Asmussen (1976b), that spindles show an increase in length following denervation.

It is difficult to assess quantitatively any change in either the size of the periaxial space, or in the thickness of the capsule, as the capsule is squashed to varying degrees, according to the amount of the surrounding intrafusal muscle fibres, by the process of whole-mounting. Nevertheless spindles without any periaxial space around the primary restoration have been seen in 8 week post-freeze material. This is consistent with the observation made by Kucera (1980b).

No attempt has been made to quantify the number of spindles present in reinnervated muscles, but the number of analysable spindles is not equivalent to the actual number of spindles present , as they are sometimes unavoidably lost or damaged in the teasing process, and therefore cannot be considered analysable. The number of analysable spindles is however at least 50% of the normal complement. This is not consistent with the observations made by Arendt & Asmussen (1976a), that the number of spindles is reduced by up to 50% in muscles which have been denervated.

One explanation for the results of Arendt & Asmussen (1976a) may be that the periaxial space of many spindles had been reduced to an extent that they were missed in the counting procedure.

Comparison with previous studies

The observations made from the deafferentation and nerve section experiments (Chapter 4, Section 2) would appear to contradict the assertion made by Ip, Vrbova & Westbury (1977) that the afferent ending does not elaborate normally in the absence of motor innervation. However, the hypothesis of " site-type " specificity breakdown, derived from this study, allows another interpretation of their observations.

In their experiments, the ventral roots were sectioned, but it has

been established (Chapter 4, Section 2) that crushing the ventral roots slows down the rate of regeneration of the remaining sensory nerves. It is probable that sectioning the ventral roots will delay the regeneration of the sensory axons for at least as long. This would mean that the muscle spindles which are described, were being innervated after an estimated 48 days D.T. (6 days recovery, 27 days delay in growth time, 14 days growing time). The exact date at which axons returned to spindles is not known, as the first sample was taken at 56 days. As has been demonstrated (Table 5.1), by 48 days D.T., the ability of the primary site to interact with a Ia axon to produce a primary ending, has reduced considerably.

Such an explanation would predict that the formations adopted by the reinnervating Ia axons would be of similar form to the spindles in the cut and 8 week freeze studies (Chapter 4, Sections 1 & 4). In fact, their figure 1.d shows a very close resemblance to Plate 4.9 A and their figure 1.e to Plate 4.9 A & B.

This explanation would be consistent with other observations which they report. They noticed that some primaries regained a normal appearance while others did not. This is consistent with the reinnervation occurring over a period when site-type specificity is beginning to break down.

They also noticed that the degree of restoration appeared to be independent of the time after crush, again not inconsistent with the hypothesis of site-type specificity breakdown, as it has been shown that abnormalities once formed can persist for long periods.

The examples of good sensory innervation with poor intrafusal motor innervation agree with the proposed hypothesis too, but force the assumption from the theory of Ip, Vrbova & Westbury (1977) that it is the degree of extrafusal reinnervation which mediates the success of primary restoration.

The authors interpret this as meaning that functional activity is needed for normal primary elaboration. The only indication however that this was the case, would be if there was a correlation between the state of the extrafusal endplate reinnervation in the antagonist muscle, and the state of restoration of the primaries. The extrafusal innervation was however assessed in the muscles from which the spindles were taken, and they would give a reliable index of the state of reinnervation of the antagonist, as there is an indication that all the ventral roots were not totally ablated (" where section of the ventral roots was more complete " (Ip et al., 1977)).

Another explanation of their observations is that, in those muscles where the motor innervation was successfully restored, this was due to there being less damage caused at the site of the injury, and thereby the sensory axons regenerating more quickly, reinnervating before the site-type specificity had diminished.

The results from the previous study of Ip & Vrbova (1973) are difficult to assess in terms of this hypothesis because no calculation can be made to estimate the length of denervation time, and the fact that both animals were kittens may involve other unknown factors.

Therefore, although the sensory innervation may regenerate at a slower rate than the motor, this study has shown that the observations made by Ip et al. (1977) are capable of being explained in terms of a theory other than theirs of the effects of the efferent innervation.

Brown & Butler (1976) commented that in a response to stretch from reinnervated spindle primaries, some units fired only during the dynamic phase of stretching. Similar observations were made by Ip, Vrbova & Westbury (1977), where they found that 66% of spindles showed abnormalities only in the maintained part of the response.

It was suggested in the Brown & Butler study (1976) that abnormal responses may be due to early " incomplete " innervation, as abnormal behaviour was commoner in animals examined early after the crush. Such effects as described by both groups of workers might be caused by a deficit in the innervation by the primary ending to the bag₂ and chain fibres. The crush study (Chapter 3) has however shown that no component of the intrafusal bundle is consistently innervated to the exclusion of others. This implies that the abnormalities in the responses are due to some other effect.

The work of Scott (1982) and Hyde & Scott (1983) on the responses of reinnervated spindles, has led them to conclude that the abnormalities in the response are indeed due to an immature transduction mechanism. They suggest that the abnormalities can be accounted for by a subtractive reduction in the firing frequency. This is attributed by them to an increase in the pace-maker threshold. As the ending matures, the threshold drops and the response loses its abnormalities.

The arguments presented by Brown & Butler (1976) to show that there was some degree of specificity of innervation was, by their own admission, not very strong. The results however of the studies presented in this work do suggest strongly that the reinnervation of afferent axons to spindles shows a marked site-type, and thus functional specificity, although not necessarily an original -terminal specificity.

The strongest evidence cited by Brown & Butler in favour of specificity is based on their observations from the gamma innervation, namely the consistency of static or dynamic response. The results from their crush studies are only of peripheral interest in this respect, as regeneration down the original endoneurial tube does not provide a test for specificity of innervation. This is not so in the case of the cut studies

where any form of innervation is theoretically possible. In these experiments, 11 out of a possible 15 gamma axons showed consistency of function on more than one presumed spindle primary.

This present study does not provide any compelling evidence for rejecting Brown & Butler's (1976) hypothesis that static and dynamic axons return to their original sites, but evidence has been found which suggests that former p_2 and p_1 sites can be innervated by axons of a type other than that with which they were originally innervated.

If motor nerve sprouting is induced indiscriminately within the motor complement by vacant sites, and there is some evidence that this occurs, then specificity of motor innervation is unlikely to occur.

The apparent inconsistency between this and the physiological study of Brown & Butler (1976) may be explained by the possibility of " weak innervation of inappropriate sites " not being detected.

The hypothesis derived from the studies presented here, that of spindle site-type specificity breakdown with absence of Ia axons could usefully be investigated further, by enlarging upon the pilot studies described here, that is minimising the time of denervation and maximising it.

It would also be useful to observe spindles after very long periods of reinnervation time following nerve crush and section, in order to see if normal-looking primaries are ever restored.

Using these studies as a baseline, it would be possible to assess the efficacy of different surgical techniques used in clinical nerve repair.

By combining physiological recording with the staining technique described here, it would be possible to look at the response and histology of one particular spindle. Any abnormalities in the responses may be reflected in the histology and this could improve the understanding of the normal function of the spindle in relation to its innervation.

Summary

This study has been the first to investigate both the sensory and motor reinnervation of the muscle spindle and the reinnervation of GTO organs, using a whole-mount method, in the terms of the now accepted normal distribution of primaries, secondaries, p_1 plates, p_2 plates and trail endings over the intrafusal bundle. This reinnervation process has been studied following the different lesions of crush, ^{cut} nerve freeze and a pilot study of nerve entry crush. As expected, the nature of the lesion greatly affected the success of restoration.

The results of these studies indicate that assumptions made as to the nature of axons forming recognisable endings, were correct. They also indicate that for the sensory component, site-type specificity was effective after short periods of denervation, but there were indications that original-terminal specificity did not operate.

It was found that site-type specificity broke down following prolonged periods of denervation, and the process may be speeded up by the presence of alpha motoneurons on the intrafusal bundle.

This breakdown in site-type specificity could account for the observations made by Ip, Vrbova & Westbury (1977), from which they concluded that sensory elaboration is dependent upon the state of motor innervation.

The fact that there is no consistent observation of a deficit in primary elaboration to any one component of the intrafusal bundle, indicates that the reported abnormalities of the response are not due to errors in the distribution of the ending in the crush study. These may be due to an immature transduction process, as abnormalities of response decrease with time.

Some observations could indicate that sensory nerve sprouting is occurring.

No compelling evidence has been found to point to a specific innervation by the motor component, and in fact there are indications that this is not the case.

Evidence has been presented to explain the increased beta-static innervation (alpha invaders), found in material from both crush and section experiments.

Both intrafusal and extrafusal motor nerve sprouting have been reported, and evidence has been put forward which indicates that intrafusal muscle fibres, in particular bag₁ fibres, may split following denervation.

This work has provided the results against which experiments devised to assess the efficacy of surgical procedures can be measured. It has also provided the means by which the spindles involved in these experiments can be stained.

APPENDIX

TECHNIQUES.

a) Holmes Silver.

The calculations of Growth Rates, Reorganization Time and Reinnervation as defined in Chapter 4.1 were determined from longitudinal sections of nerves stained using the Holmes Silver technique as follows.

- 1) Lengths of nerve containing the operation site were removed and fixed in Bouin fluid, and left for 2 days.
- 2) The nerves were dehydrated through alcohols and vacuum-embedded in Carbowax.
- 3) Longitudinal sections of the nerve were taken on a microtome, 35μ thick.
- 4) Sections were dried on to albumen-coated slides.
- 5) The sections were then stained using the Holmes technique, and counterstained using Gold Chloride.
- 6) The sections were then examined for Growth cones, and measurements were taken using a micrometer eye piece.

For details of experimental design, see Chapter 4.1.

b) Freeze equipment.

The nerve-freeze experiments were performed in order to produce a nerve injury which did not damage the endoneurial tubes. The ideal freeze injury would be of very limited extent, so that scar tissue would be minimised, and applied very quickly so that large ice crystals would not rupture the endoneurial tubes.

The cryogenic apparatus supplied by Kryospray consisted of a stainless steel cylinder that contained liquid nitrogen. When sealed the evaporating nitrogen caused an increase in pressure that forced liquid nitrogen into a cryode when a valve was operated.

The cryodes supplied were not capable of producing a narrow linear injury because they had very large diameter endpieces, and a special cryode was constructed to meet these requirements.

It consisted of copper "microbore" tubing, one end of which was formed into a narrow vertical probe which could be inserted underneath a nerve and used to withdraw the nerve from underlying tissues. This procedure allowed minimum damage to the surrounding tissues, and froze the nerve only in a very narrow band. The liquid nitrogen was let through the cryode by operating the valve on the stainless steel cannister. When the nerve was seen to be frozen across its width, the supply of liquid nitrogen was switched off, and the cryode with the nerve still adhering was lowered onto the muscles beneath to speed melting. When the nerve was no longer frozen to the cryode, the latter was withdrawn carefully. See Fig.A.1.

c) Anaesthetic box.

Operations were initially carried out using Sagatal (May & Baker) anaesthesia. However some problems were encountered concerned with induction and recovery. To make the operative procedure more reliable it was decided to adopt Halothane (Fluothane ICI) as the anaesthetic. This was usually used after a very low initial dose of Sagatal.

A Fluotec Mark 2 calibrated vaporizer (Cyprane Ltd.) was used with 95% oxygen and 5% CO₂ gas mixture.

However a recent D.H.S.S. report on the use of Halothane in operating theatres declared that exposure to Halothane was a potential health hazard.

It was decided to construct a scavanging system to remove the excess Halothane. This consisted of a perspex box.(Figs. A.2&3) One face of the box was located by grooves which allowed it to be withdrawn. At the lower edge of this piece was a semi-circular aperture. The animal was positioned with its head in the box, and held by lowering the side so that the aperture sealed around the animal's neck. This piece could be replaced by another with a different size of aperture to accommodate a different size of animal.

On the opposite wall of the box was the inlet valve. This took the form of a hinged flap of perspex.(Figs. A.2&3)The valve would only operate when there was negative pressure within the box, ensuring that no Fluothane could escape, and it also gave an immediate visual indication that the extraction system was in operation.

The top of the box was permanently fixed. It had two holes in it : one for the extraction pipe and the other for the anaesthetic gas.

The tubing for the anaesthetic gas was connected to the vaporizer and led to a mask that was placed over the animal's head inside the box. The tubing for the extracted gases was connected to another box. This contained a Secomak 574 fan (Secomak Air Products Ltd.).

The fan box had a sealed exhaust tube leading outside the operating theatre. The tube from the anaesthetic box was fixed through one side of the fan box, but not directly to the fan input. the opposite side of the fan box was patent and positioned next to the evaporator. (Figs. A.2&3)

As the anaesthetic box worked by virtue of the negative pressure within it, as opposed to a high current of air alone, the extra volume that the fan could move was accommodated by the open side of the fan box acting as a scavenging system around the vaporizer. This also allowed the fan to work at its most effective rate.

The anaesthetic gas was administered as required, and when this method was used no animals were lost as a result of anaesthesia.

d) Plastic embedding.

Nerves taken for plastic embedding were treated as follows.

Fixation: The tissue was removed and placed immediately in the fixative outlined below, in a refrigerator. The fixative was changed after one hour, and again after two hours, and then left overnight.

5mls	25%	Gluteraldehyde
25mls	0.2M	Cacodylate
20mls		Distilled water

Wash: The nerve was washed for half an hour in 0.1 Cacodylate.

Post-fixation: The tissue was postfixed in the following solution for two hours in the refrigerator.

25ml	4%	Osmium
25ml	0.2M	Cacodylate

Wash: Washing took place for 30 minutes with two changes of solution.

25ml	0.2 M	Cacodylate
25ml		Distilled water

Dehydration: The material was dehydrated through a series of alcohols: 70%, 80%, 90%, 95%, Absolute, with three changes at each stage, and for a total of 15 minutes at each concentration.

Embedding:

The nerves were then embedded in Araldite using the following procedure.

25ml Propylene Oxide	30 minutes with two changes
25ml Absolute Alcohol	
50ml Propylene Oxide	30 minutes with three changes
25ml Propylene Oxide	30 minutes
25ml Araldite	
Araldite	Two changes before the addition of accelerator and curing in a 45° C oven overnight.

e) Electron Microscopy.

Tissue embedded in Araldite was sectioned on a Reichert U 2 ultramicrotome. Sections 1μ thick were examined under a light microscope, having been stained with a 1% solution of toluidine blue in 1% borax. Subsequently sections $\approx 100\text{nm}$ were taken for E.M. They were double stained with uranyl acetate followed by lead citrate and examined with an AEI 801 electron microscope, at an accelerating voltage of 60,000 V., and photographed on Kodak 4489 E.M. film.

f) Photography.

Silver preparations were photographed on a Zeiss Ultraphot using either Ilford 120 HP4 or Kodak 35mm Pan F. film.

g) Tracing.

The tracing of axons within the spindle was achieved by two methods. The first of these was to create a montage from photomicrographs of the relevant area before transferring the course of the axons onto polyester film. The second method was to use a Nikon microscope upon which was mounted a Hitachi T.V. camera; the microscope image being relayed to a 9" black and white monitor. A sheet of perspex was placed in front of the screen, and the image traced onto polyester film which was subsequently arranged into a montage. This arrangement avoided the conventional photographic process altogether.

h) Silver Staining.

The technique finally adopted for the silver staining of whole preparations is outlined below.

Method:

- 1) Animals are killed with an intraperitoneally injected overdose of Pentobarbitone sodium (Sagatal, May & Baker).
- 2) Dissection is started within three quarters of an hour post mortem.
- 3) Muscles are attached by nylon thread to polystyrene labels, and suspended from a polypropylene ring. This ring fits into the neck of a 500ml screw-top bottle, so that the muscles hang freely in the contents of the bottle, while allowing the bottle top to be screwed in place. The muscles are moved between stages by transferring the ring to the different bottles.
- 4) Muscles are fixed for a minimum period of five days. Further fixation does not improve the staining, but may cause the muscle to disintegrate during subsequent stages.

Fixative:

10g chloral hydrate
450ml 95% alcohol
500ml distilled water
Adjust to pH 1.7 with conc. HNO_3 (10ml)

- 5) Wash the muscles for 24h at the rate of one litre per hour. Keep the washing bottle cool with running tap water.

Washing solution:

30 litres distilled water

6g $\text{Al}_4 (\text{SO}_4)_3$

Adjust to pH 9 with saturated NaOH.

- 6) Place for 30h in Ammoniacal alcohol:

To 95% alcohol add 880 Ammonia until pH 9 is reached.

- 7) Coat with agar:

Dissolve 10gm of agar in one litre of distilled water. Bring to the boil. As the solution cools ($< 50^\circ \text{C}$), dip the muscles repeatedly into it until a layer of agar approximately 2mm thick surrounds each muscle. Leave to gel.

- 8) Incubate at 37°C for 7 days in 1.5% silver nitrate in a shaking water bath in the dark.

- 9) Remove the agar coat with paper tissues.

- 10) Reduce for 48h.

Reducer:

20gm Quinol

250ml 98-100% Formic acid

750ml Distilled water

- 11) Rinse in Distilled water.

12) Place in glycerine for a minimum period of two days.

Change the glycerine at least twice.

i) Teasing.

After softening in glycerine muscles were teased under an Olympus dissecting microscope, by compressing them beneath a glass slide and subsequently dissecting and removing muscle spindles and tendon organs with a pair of mounted syringe needles.

The preparations were completed by mounting them in glycerine on glass slides under circular coverslips and then ringing them with tar.

j) Operating procedure

All operations were performed aseptically. The animals were anaesthetized with an initial intra-peritoneal injection of sodium pentobarbitone (Sagatal: May & Baker Ltd. ; 40mg /kg), and were then maintained under Halothane.

After the operation, the skin was stitched with surgical silk and the wound dusted with anti-biotic powder and sealed with Nobecutane.

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